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EFFECTS OF THE PANCREAS AND THE ADRENALS UPON PRODUCTION OF NITROGEN STORAGE WITH PITUITARY PREPARATIONS

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NUMEROUS STUDIES indicate that the effect of anterior pituitary preparations upon metabolism of protein and carbohydrate is greatly influenced by the pancreas. In experiments conducted in this laboratory upon normal fed bitches, single large injections of a growth preparation induced storage of nitrogen, sudden gains in weight, little or no glycosuria or hyperglycemia, and a negligible rise in blood fat. In depancreatized bitches receiving constant diets and uniform daily doses of insulin the effects of the same preparation were practically reversed. Losses of weight and nitrogen occurred, as well as greatly increased glycosuria, hyperglycemia, lipemia, and ketonuria (1).

If the response of normal animals depends upon ability to increase or mobilize their insulin supply, one should be able to produce the same response in depancreatized animals by raising the insulin dosage when the growth preparation is injected. Such experiments form the first part of the present study. They have been more completely successful than previous ones (1) and furnish good evidence that the growth preparation which we used stimulates insulin production or mobilization in normal animals. That the growth preparation produces nitrogen storage by this mechanism would follow if its effects upon nitrogen storage in normal fed animals could be duplicated by injecting insulin. Experiments on this point yielded negative results (1). The alternative view, that an increase in available insulin is a necessary condition for production of optimal storage of nitrogen by a growth preparation, remained.

Further evidence for this interpretation would be at hand if one could produce some storage of nitrogen with a pituitary preparation in fed depancreatized animals receiving a constant dose of insulin. This was the second objective of our study. In pursuing it we varied the dosage and method of administering pituitary preparations, and used preparations which differ greatly in the degree to which they accentuate pancreatic diabetes. We made unsuccessful attempts to alter one of the preparations in the hope of decreasing its diabetogenic properties without destroying its effect upon nitrogen storage. Finally we removed the thyroid or the adrenals from the depancreatized animals, to diminish calorigenic and diabetogenic effects. It was hoped that this might enable us to produce nitrogen storage with the insulin supply constant.

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EXPERIMENTAL PROCEDURE

Animals and general conditions. The animals were normal adult bitches. Special attention was given to conditions which are essential in balance studies. The quantity of food which each animal would consume regularly was determined in long preliminary observations which also brought each subject into equilibrium with its diet. In the case of depancreatized animals the food and the insulin were usually given in equal portions, at about 8:30 A.M. and 5:30 P.M., and no experiments were begun until the feces nitrogen was less than 10 per cent of the intake. Among other conditions may be mentioned adequate recovery periods following operations, satisfactory control of deficiencies produced by operations, and good renal function. The urine volume, which was recorded daily in all experiments, is a sensitive indicator of a departure from normal renal function in dogs receiving a constant high protein diet.

Diets and methods. Diet 1 consisted of beef heart, cracker meal, and bone meal. *Diet 5* was a dry mixture of casein, cracker meal, corn oil, yeast, and salt mixture. Karr's salt mixture (2) without added iodine was used in all diets. *Diet 8* consisted of trimmed beef heart, raw pancreas trimmed free of fat, casein, and salt mixture. Constancy of this mixture was maintained by the method previously described (1). Details of other diets are given in the tables. All animals received 15 drops of haliver oil daily. The thyroparathyroidectomized animal received 1 cc. of viosterol and haliver oil daily, and for some time after pancreatectomy required 5 to 10 times as much.

The weight of each animal was determined daily, after catheterization, to quarter-pound accuracy. Urines were collected under toluene and all 24-hour periods were terminated by catheterization. Stools were collected in hydrochloric acid, in 3-day periods. Blood specimens were drawn during the postabsorptive period. All nitrogen balance determinations are based upon complete analyses of food, feces, and urine. The analytical methods were the same as in preceding studies of this series.

Endocrine preparations. Insulin and protamine insulin[1] were used to control pancreatic diabetes. To prevent adrenal insufficiency, an adrenal cortex extract[2] was used. The pituitary preparations included growth and lactogenic preparations[2] and a preparation of prolactin.[3] We are indebted to Dr. J. P. Schooley of the Difco Laboratories for having assays for lactogenic activity made upon the three preparations simultaneously in the same laboratory. Since normal animals require about two weeks to complete a cycle of nitrogen storage and loss following a large injection of a growth preparation, injections were given at shorter intervals only in very few instances.

RESULTS

The effects of injecting the growth preparation and increasing the insulin dosage in depancreatized animals will be considered first. The experiment, data for which are shown in table 1, was made after two preliminary trials had established the relative amounts of growth preparation and insulin which would have approximately compensating effects in this particular animal. When 100 mg. of the growth preparation was given, it was necessary to increase the insulin dosage to the very high level of 85 U daily. This amount still permitted a rise in the postabsorptive true blood sugar from 39 to 180 mg. per 100 cc. of blood, but the glycosuria was slight and the effect on blood fat negligible. Under these circumstances there was a gain in weight, and marked storage of nitrogen. The time of maximum storage and the subsequent loss simulated the response of intact animals to the growth preparation alone.

[1] The insulin and protamine insulin were supplied by Eli Lilly & Co., Indianapolis, Ind.

[2] The adrenal cortex extract (Eschatin), the anterior pituitary growth (Antuitrin-G) and lactogenic (Antuitrin-L) preparations were supplied by Parke, Davis & Co., Detroit, Mich.

[3] A preparation of prolactin was supplied by the Difco Laboratories, Detroit, Mich.

TABLE 1. EFFECT OF INJECTING A LARGE SINGLE DOSE OF A GROWTH PREPARATION AND SIMULTANEOUSLY INCREASING THE INSULIN DOSAGE, IN A FED DEPANCREATIZED DOG

Date	Procedure	Insulin per Day	Weight	Urine N per Day	Nitrogen Balance per Day	Urine Sugar	Blood Sugar	Blood Fat
1940		units	kg.	gm.	gm.	gm.	mg./100 cc.	mg./100 cc
12/4–9	6-day control	25	10.75	15.22	−1.02	trace	39	868
12/10	100 mg., growth preparation	85	10.77	13.72	+0.59	trace	180	954
12/11		85	10.88	11.20	+3.11	5.0	132	979
12/12		75	10.88	12.90	+1.41	8.1	60	
12/13		45	10.88	13.12	+1.39	5.0	38	926
12/14		25	10.99	14.61	−0.10	trace		892
12/15		25	10.88	14.67	−0.16	trace	68	
12/16–18	3-day control	25	10.65	15.61	−1.10	trace		

Ten U of protamine insulin was injected every morning; the remainder of the total dose recorded consisted of ordinary insulin, injected in two equal portions, mornings and evenings. *Diet 8*, plus 25 gm. of sucrose daily, was fed. The nitrogen intake was 15.57 gm. per day, and the feces nitrogen varied between 1.02 and 1.47 gm. daily.

In table 2 are recorded data of similar experiments performed upon another depancreatized animal before and after thyroparathyroidectomy. Doubling the basal insulin dosage when 200 mg. of the growth preparation was injected permitted definite nitrogen storage, even though the glycosuria was greatly increased. In a previous study (1) a similar result was regularly obtained in a thyroidectomized-depancreatized animal, but not in a depancreatized animal with the thyroid present. In the present experiment nitrogen storage was induced in a depancreatized dog with the thyroid intact, and was not improved by thyroparathyroidectomy. In fact, when the experi-

TABLE 2. EFFECT OF INJECTING LARGE SINGLE DOSES OF A GROWTH PREPARATION AND SIMULTANEOUSLY INCREASING THE INSULIN DOSAGE, IN A DEPANCREATIZED DOG, BEFORE AND AFTER THYROPARATHYROIDECTOMY

Date	Procedure	Insulin per Day	Weight	Urine N per Day	Nitrogen Balance per Day	Urine Sugar	Blood Sugar	Blood Fat
1940		units	kg.	gm.	gm.	gm.	mg./100 cc.	mg./100 cc.
10/8–13	6-day control	24	15.78	27.46	+0.49	5.3	72	922
10/14	200 mg. growth preparation	48	15.62	29.23	−1.52	30.0		
10/15		48	15.62	27.77	−0.06	47.9	220	847
10/16		48	15.97	25.66	+2.05	32.9		
10/17		48	16.19	24.68	+3.43	6.6		
10/18		36	16.30	26.88	+1.23	trace	80	731
10/19		24	16.19	27.76	+0.34	trace		
10/20–25	6-day control	24–20	16.06	29.33	−1.34	4.3		862
10/28	Thyroparathyroidectomy							
12/13–15	3-day control	30	17.11	28.07	+0.67	9.2	114	1216
12/16	200 mg., growth preparation	60	17.57	28.34	+0.72	trace	250	1272
12/17		60	17.44	27.15	+1.91	59.6	258	
12/18		60	17.67	27.72	+1.34	70.0		934
12/19–21	3-day control	60–45	17.74	30.51	−1.09	39.5	86	1087
12/22–24	3-day control	30		29.27		14.8		

Twenty-four U of protamine insulin was injected daily; increases in the total insulin dosage were made by injecting plain insulin, in equal doses, morning and evening. *Diet 8*, plus 50 gm. of sucrose daily, was fed. Before 10/25/40 the nitrogen intake was 30.46 gm. per day, and the stool nitrogen 2.15 to 2.80 gm. per day. Thereafter the nitrogen intake was 31.14 gm. daily, and the stool nitrogen 1.72 to 2.40 gm. daily. Significant ketonuria occurred only on 10/15/40, when the 24-hour excretion of acetone, diacetic, and beta hydroxybutyric acid amounted to 274 mg., calculated as acetone.

ment was repeated after this operation (table 2, December 13 to 24), the storage of nitrogen was somewhat smaller and the glycosuria somewhat greater.

In the second part of our study we observed the comparative effects of three pituitary preparations administered to normal and to depancreatized animals, both groups receiving constant diets, and the latter receiving uniform daily doses of insulin. Details of one experiment are given in table 3. After a preliminary control period of 9 days, 40 mg. of prolactin was given daily for 5 days. Urine nitrogen decreased, and the nitrogen balance was doubled. For the 5-day period the increase in the balance

TABLE 3. EFFECT OF INJECTING A PROLACTIN PREPARATION IN A DEPANCREATIZED DOG RECEIVING A CONSTANT DIET AND A UNIFORM DAILY DOSE OF INSULIN

Date	Water Intake	Urine Volume	Weight	Urine N per Day	Nitrogen Balance per Day	Urine Sugar	Blood Sugar	Blood Fat	Subcutaneous Injections
1940	cc.	cc.	kg.	gm.	gm.	gm.	mg./100 cc.	mg./100 cc.	
10/12	1020	750	10.53	13.11					25 U of ordinary insulin daily throughout the experiment.
10/13	1020	850	10.53	13.35					
10/14	1080	890	10.42	13.05					
10/15	1070	740	10.53	12.99		20.0	302	772	
10/16	1060	750	10.53	12.89		17.2			
10/17	1000	710	10.53	13.04		10.0			
10/18	1240	900	10.65	13.93		11.1	280	750	
10/19	1010	630	10.65	13.07		8.0			
10/20	980	680	10.65	12.78		5.5			
Average	1053	766	10.56	13.13	+0.88	12.0			
10/21	950	600	10.77	11.55		15.3			40 mg. prolactin
10/22	1320	950	10.88	11.53		29.3	370	840	40 mg. prolactin
10/23	1370	870	10.77	12.92		26.7	354	862	40 mg. prolactin
10/24	1200	890?	10.77	11.79		36.4			40 mg. prolactin
10/25	1270	890?	10.88	12.78		33.9			40 mg. prolactin
Average	1222	840	10.81	12.11	+1.76	28.3			
10/26	1040	740	10.77	14.02		36.4			
10/27	800	510	10.65	14.37		26.7			
10/28	870	510	10.65	14.70		14.8			
10/29	920	550	10.77	14.55		12.8	219	803	
10/30	840	520	10.77	14.30		10.6			
10/31	740	520	10.53	14.86		16.4	222	859	
11/1	800	520	10.53	14.45		15.6			
Average	859	553	10.67	14.46	−0.14	19.0			

The animal received *diet 8* plus 25 gm. of sucrose daily. From 10/12 to 10/25 the nitrogen intake was 15.23 gm. daily; thereafter 15.61 gm. daily. The feces nitrogen varied between 1.09 and 1.48 gm. per day. The water intake recorded includes water present in the food or available from it by oxidation.

amounted to 4.40 gm. of nitrogen, which is the storage recorded for this experiment in table 4. This storage occurred in spite of an increase in the glycosuria. It was accompanied by effects seen in normal animals—a rise in water intake exceeding the rise in urine volume, and an increase in weight. That the postabsorptive blood sugar was high, both in relation to the glycosuria and to the insulin dosage, was no doubt due to the fact that the diabetes was controlled with plain insulin alone, given at 9 A.M. and 5 P.M., and that the diet included sucrose. Blood specimens were taken at about 8:30 A.M., and the high blood-sugar level observed had probably been reached only a short time previously.

In table 4 there are summarized the more important findings in sixteen experiments similar to the one presented in table 3. The data of the first nine experiments show the results in normal bitches on complete and adequate diets. Single large injections of the pituitary preparations were given. Nitrogen storage was calculated from the time of the injections until the urine nitrogen returned to the control level. The lactogenic preparation and prolactin both assayed approximately 10 I.U. per mg., while the growth preparation had only one-fifth to one-tenth of this lactogenic

TABLE 4. EXPERIMENTS IN WHICH NORMAL DOGS, OR DEPANCREATIZED DOGS RECEIVING CONSTANT DOSES OF INSULIN, WERE GIVEN GROWTH OR LACTOGENIC PREPARATIONS

Dog Number	Condition	Preparation	Dose	Nitrogen Stored	Calorigenic Effect	Increase in Glycosuria	Diet Number
			mg.	*gm.*	*cal.*	*gm.*	
14	Normal	Growth	200	+12.24	713–1039		5
20	Normal	Growth	200	+14.35	in previous		8
11	Normal	Growth	200	+ 8.80	experiments		1
11	Normal	Lactogenic	200	−11.52			9
11	Normal	Lactogenic	200	+ 4.95			5
14	Normal	Lactogenic	200	+10.05	581		5
14	Normal	Prolactin	40	+ 5.46	113		5
14	Normal	Prolactin	200	+ 7.23	177		5
11	Normal	Prolactin	200	+ 2.49			1
20	Depancreatized	Prolactin	40	0.00		0	8
25	Depancreatized	Prolactin	200	+ 4.65		59 in 2 days	8
25	Depancreatized	Prolactin	5×40	− 1.56		35 in 6 days	8
12	Depancreatized	Prolactin	5×40	+ 4.40		106 in 6 days	8
12	Depancreatized	Prolactin	200	− 0.60		61 in 6 days	8
12	Depancreatized	Lactogenic	140	0.00		29 in 3 days	8
25	Depancreatized	Lactogenic	5×40	− 1.74?		29 in 6 days	8

In three instances the pituitary preparations were injected in five 40 mg.-doses on successive days; in the other experiments single large injections were given.

activity. The growth preparation however produced the largest storage of nitrogen. A few determinations of the calorigenic effect are also included, and these indicate no correlation with lactogenic activity. In determining the calorigenic effect we used animals which were already well-trained and determined the basal metabolism daily from the time of the injection until the metabolic rate returned to the control level, so that the total number of additional basal calories could be calculated.

The lactogenic preparations, while they produced less nitrogen storage than the growth preparation in normal animals, had two desirable properties. In depancreatized animals they increased the glycosuria and the level of blood fats much less than did the growth preparation, and they did not cause anorexia or vomiting as commonly as the growth preparation when administered without additional insulin. In two instances definite storage of nitrogen was produced in depancreatized animals receiving a constant dosage of insulin; in two others no storage occurred, and in three there were negligible or questionable losses. Similar experiments with the growth preparation, omitted from table 4, always terminated in refusal of food or vomiting, or in nitrogen loss. While experience with the lactogenic preparations was more favorable, it is none the less evident that the goal of producing nitrogen storage in depancreatized animals with the insulin supply constant was only reached in a few instances.

Experiments upon depancreatized-adrenalectomized dogs proved to be very difficult, apart from the mortality of successive operations. Perfectly regular eating habits

and constancy of the insulin requirement could only be re-established by maintaining the animals for a long time after adrenalectomy. One of the two animals in which we did this at first required adrenal cortex extract but later was able to do without it. Throughout its postoperative life it had, however, the very low insulin requirement and extreme susceptibility to insulin shock characteristic of adrenalectomized-depancreatized animals. After pancreatectomy, and before adrenalectomy, the insulin requirement was high, and no pancreatic tissue was found in a careful autopsy. It was found, however, that in attempting to pass a ligature around the lower pole of the left adrenal we had ligated off a small piece of cortical tissue which remained in

TABLE 5. EFFECTS OF INJECTING A GROWTH PREPARATION IN A DEPANCREATIZED, PARTIALLY ADRENALECTOMIZED DOG REQUIRING VERY LITTLE INSULIN

Date	Weight	Urine N per Day	Nitrogen Balance per Day	Urine Sugar	Blood Sugar	Blood N.P.N.	Subcutaneous Injections
1941	kg.	gm.	gm.	gm.	mg./100 cc.	mg./100 cc.	
9/14	13.6	17.71		trace	152	42.7	
9/15	13.6	18.70		trace	224	39.4	
9/16	13.5	18.64		trace	242		
Average		18.35	+0.50				
9/17	13.5	18.80		18.3	314	40.1	100 mg., growth preparation
9/18	13.5	18.48		47.0	296	39.1	
9/19	13.4	19.99		20.5			
Average		19.09	−0.31	28.6			
9/28	13.7	19.43		10.4	50		
9/29	13.7	18.95		12.5	22	30.0	
9/30	13.7	18.90		neg.	84	39.8	
Average		19.09	+1.44	7.6			
10/1	13.7	20.32		6.5	170	34.6	200 mg., growth preparation
10/2	13.8	19.62		27.2	216	34.1	
10/3	13.8	19.06		51.8	234	41.3	
Average		19.67	+0.82	28.5			

From 9/14 to 9/19 the animal received 4 U of protamine insulin and 2 U of plain insulin daily; thereafter, 5 U of protamine insulin and 2 U of plain insulin daily. The diet consisted of 250 gm. of raw pancreas, 100 gm. of casein, 25 gm. of calcium phosphate, 10 gm. of salt mixture, 5 gm. of yeast, and 15 drops of haliver oil daily. The nitrogen intake was 19.78 gm. daily before 9/19 and 21.74 gm. daily thereafter; the stool nitrogen varied between 1.00 and 1.25 gm. per day.

the animal. Thus we had experimental data on a depancreatized, partially adrenalectomized animal requiring no adrenal cortex extract and very little insulin, and a depancreatized, completely adrenalectomized animal, from which the thyroid and parathyroids had also been removed in the course of previous experiments. The latter animal, receiving a liberal diet and dosage of cortical extract, required somewhat less than 15 U of insulin daily. Withdrawal of cortical extract was followed within a few days by a rise in non-protein nitrogen of the blood, weakness in the hind legs, and refusal of food. The autopsy revealed neither adrenal nor pancreatic tissue.

Experimental data on the first of these animals are recorded in table 5. Nearly complete adrenalectomy made it possible to give this depancreatized animal, receiving only 6 U of insulin daily in the first experiment and 7 U daily in the second, large single does of growth hormone without causing anorexia or vomiting. Considerable glycosuria still occurred in both experiments however, and nitrogen loss was just detectable. Results in the other animal are shown in table 6. Here at last definite nitrogen storage was produced in two successive experiments with the growth hormone preparation with the insulin supply constant. The greatest effect upon urine nitrogen,

as in normal animals, occurred during the second 24-hour period following injections. The amount of storage was not of the same order as in normal animals, yet there was no doubt concerning its occurrence. In the first experiment (table 6, April 28) effects upon the glycosuria, blood sugar, and blood fat are all evident, despite absence of the adrenals, but they are much less pronounced than in simply depancreatized animals. In the second experiment (table 6, May 21) the urine sugar had been reduced to traces and the true blood sugar to 30 mg. per 100 cc. when the growth preparation was in-

TABLE 6. RESULTS OF INJECTING A GROWTH PREPARATION IN A DOG WITH PANCREAS, ADRENALS, THYROID, AND PARATHYROIDS REMOVED, THE DIET AND INSULIN DOSAGE BEING CONSTANT

Date	Water Intake	Urine Volume	Weight	Urine N	Nitrogen Balance	Urine Sugar	Blood Sugar	Blood Fat	Blood N.P.N.	Subcutaneous Injections
1941	cc.	cc.	kg.	gm.	gm.	gm.	mg./100 cc.	mg./100 cc.	mg./100 cc.	
4/22	1000	450	17.21	16.20		25.2				
4/23	1030	900	17.13	17.48		20.0				
4/24	980	840	16.88	17.42		25.2	52	570	36	
4/25	960	600	16.99	16.94		12.8				
4/26	870	770	16.88	16.82		14.3				
4/27	890	430	16.88	16.34		10.0	82	600	44	
Average				16.87	−0.71					
4/28	1200	780	16.88	17.93		13.3	244	1002	32	100 mg. growth preparation
4/29	1370	1070	16.99	14.38		30.0	222	1350	34	
4/30	1390	1260	16.88	15.78		28.0				
Average				16.03	+0.15					
5/1	1220	1220	16.62	17.07		32.6	206	780	34	
5/2	1010	930	16.30	17.06		28.6				
5/3	1230	850	16.40	17.92		26.6				
Average				17.35	−1.40					
5/18	1015	390	16.99	18.06		trace				
5/19	965	540	17.12	19.71		trace	36	653	36	
5/20	1195	700	17.32	19.71		trace	30		35	
Average				19.16	+0.37					
5/21	1125	650	17.44	19.31		trace	66	592	32	200 mg. growth preparation
5/22	735	490	17.44	16.48		trace	110	615	35	
5/23	665	610	17.21	17.47		trace	76	669	30	
5/24	685	540	17.12	18.31		trace				
Average				17.89	+1.42					

The animal received 4 cc. of adrenal cortex extract, 10 U of protamine insulin, and 5 U of plain insulin daily throughout the experiment. During the first experiment, 4/22 to 5/3, the diet consisted of 250 gm. of horse meat, 125 gm. of pancreas, 25 gm. of casein, 11.5 gm. of calcium phosphate, 5 gm. of yeast, and 3.5 gm. of salt mixture, the nitrogen intake being 17.74 gm. per day. During the second experiment the diet was identical with the one described under table 5, and the nitrogen intake was 20.84 gm. per day. The feces nitrogen varied between 1.31 and 1.82 gm. per day.

jected. Only the blood sugar was affected. In both experiments the non-protein nitrogen values indicate that the fall in urine nitrogen was not due to retention of nitrogenous endproducts in the blood.

DISCUSSION

In planning these experiments we assumed that it would be possible to produce nitrogen storage with anterior pituitary preparations in depancreatized animals receiving a constant dose of insulin, if the 'diabetogenic' effects could be disposed of by altering either the pituitary preparation or the sensitivity of the animal. Although we were to some extent successful, a constant inverse relationship between nitrogen storage and glycosuria was not observed. For example (table 4), a 40 mg.-dose of prolactin produced definite nitrogen storage in a normal dog, but not in a depancreatized dog, although the glycosuria in the latter animal was not increased. Similarly, in the two instances in which nitrogen storage was observed in depancreatized dogs receiving lactogenic preparations it was accompanied by more extensive glycosuria than in several experiments in which nitrogen storage was not produced. In table 2 of this study, and in figure 5 of the previous one (1), there are instances in which storage of nitrogen was induced with a growth preparation in depancreatized dogs when the insulin dosage was moderately increased, even though a

large rise in the glycosuria occurred. When the insulin dosage is kept constant, injections of anterior pituitary preparations often fail to produce nitrogen storage in such animals even when the increase in the glycosuria is no greater than in the experiments just cited. Our experiments lend support to the idea that whether an anterior pituitary preparation will cause nitrogen storage or diabetogenic effects may depend upon the amount of insulin that the pancreas can supply (3), but they do not demonstrate that additional insulin is only needed to prevent interferences with proper utilization of carbohydrate.

SUMMARY

In fed depancreatized dogs receiving constant doses of insulin single large doses of an anterior pituitary growth preparation cause loss of nitrogen, accompanied by an increase in the glycosuria, and by hyperglycemia, lipemia, and ketosis. If the insulin dosage is increased sufficiently when the growth preparation is injected, the response of depancreatized dogs approaches that of normal ones; nitrogen is stored, and the diabetic syndrome is more or less completely absent. This is regarded as evidence that in normal animals the growth preparation stimulates production or mobilization of insulin.

Lactogenic preparations sometimes produce storage of nitrogen in fed depancreatized dogs receiving constant doses of insulin. The growth hormone preparation also produced it in a depancreatized dog which had been deprived of the thyroid, parathyroids, and both adrenals. In none of these experiments in which the insulin dosage was kept constant was the storage of nitrogen as great as in normal animals. It appears that anterior pituitary preparations do not cause nitrogen storage by stimulating the production or mobilization of insulin, but optimal storage of nitrogen will not occur unless the insulin supply is adequate. In normal fed animals it is difficult or impossible to produce nitrogen storage with insulin alone, and in depancreatized ones receiving a constant dose of insulin it is almost impossible to produce nitrogen storage with anterior pituitary preparations alone.

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GONADOTROPINS OF THE SWINE PITUITARY

I. VARIOUS BIOLOGICAL EFFECTS OF PURIFIED THYLAKENTRIN (FSH) AND PURE METAKENTRIN (ICSH)

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SEVERAL RECENT STUDIES from this laboratory offer strong support of the belief (1) that at least two gonadotropins can be isolated from extracts of pituitary glands. Preliminary evidence was obtained from experiments with extracts of hog glands digested by proteolytic enzymes permitting differentiation of gametogenic ('follicle stimulating') and interstitial cell-stimulating effects depending upon the enzyme used and the extent of digestion (2). Physicochemical methods were next employed to separate the gonadotropins from hog pituitary extracts which had not undergone preliminary enzymic hydrolysis (3). The interstitial cell-stimulating hormone as a pure protein was isolated (4). The gametogenic hormone was separated as a substance with only one apparent biological effect; however, the purest preparations contained small proportions of two contaminating impurities.

Work in the University of California (5) likewise led to the isolation of interstitial cell-stimulating hormone from pituitary glands of another mammal, the sheep. We have had the opportunity of making a limited examination of the potency and biological action of this extract believed, like our hog pituitary protein, to be a pure substance.

In this report and the two which follow it, the term thylakentrin will be used to refer to the gametogenic hormone (FSH or follicle-stimulating hormone) and the term metakentrin will refer to the interstitial cell-stimulating hormone (ICSH and, in a restricted sense only, luteinizing hormone). The basis for suggesting these names is given by Coffin and van Dyke (6).

The results of a ramified investigation of the biological action of pure metakentrin as well as more restricted and less elaborate biological experiments with thylakentrin will be described in this first report. The second report will deal completely with a simplified method of securing pure metakentrin. Physicochemical evidence of purity as well as additional biological data important for demonstrating that the protein is the hormone will also be presented. The third and last report will offer immunological evidence not only of the purity of hog metakentrin but also of its striking immunological difference from sheep metakentrin whether in crude extract or in pure form.

Unless otherwise noted, the metakentrin was the pure protein derived from hog pituitary glands and the thylakentrin, of similar origin, was about 80 per cent pure.[1]

RESULTS

Effects of Metakentrin and Thylakentrin in Hypophysectomized Male Rats

The injection of metakentrin into hypophysectomized immature male rats causes

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[1] Two inert components made up the remaining 20 per cent.

gross enlargement of the testes and the secondary sexual organs (fig. 2, 3, 4). Microscopically there is found a stimulation of the seminiferous tubules and the interstitial cells (fig. 6, 7). In the analysis of this response the following facts are pertinent: *a*), androgen injections maintain or repair the tubules but not the interstitial cells of hypophysectomized males (7, 8, 9) and *b*), the interstitial cells when stimulated secrete androgen. There is therefore justification for the assumption that metakentrin actually stimulates only the interstitial cells of the testes and that these in turn secrete the androgen which maintains and stimulates the accessory organs and the seminiferous tubules.[2]

The weight of the anterior lobe of the prostate bears a quantitative relationship to the dose of metakentrin N administered within suitable limits and is not potentiated if contaminating thylakentrin be present. This response has been proposed as a method of assaying metakentrin quantitatively (10). By this means all of our prepara-

TABLE 1. FAILURE OF METAKENTRIN TO STIMULATE GROWTH OF THE PROSTATE GLAND IN CASTRATED HYPOPHYSECTOMIZED RATS

Type of Animal	Number of Animals	Metakentrin, Total Dose	Average Weight, in mg.	
			Anterior prostate	Testes
		μg. N		
Hypophysectomized	6	5	12.8	127.2
Hypophysectomized and Castrated	5	5	8.4	
Hypophysectomized and Castrated	6	50	6.5	
Hypophysectomized and Castrated	6	0	8.3	

The animals were operated when 21 days old, injected on days 23 to 26 inclusive and killed when 27 days old.

tions of hog metakentrin have displayed constant activity in terms of micrograms of metakentrin N injected. A dose of 2 μg. of N injected into each of 30 males produced anterior prostates with an average weight of 12.75 mg. or an increase of 107 per cent over those of 57 untreated controls. We have found this dose most satisfactory as a standard in comparing the activity of numerous preparations during purification procedures. Larger doses (e.g. 360–500 μg. of N) of the metakentrin preparations were also injected into hypophysectomized females to exclude the presence of thylakentrin.

Sheep ICSH[3] has about the same potency as hog metakentrin in stimulating androgen production in hypophysectomized immature male rats. In a comparative assay at 2 μg. of N the average weights in milligrams (10 animals in each group) of the anterior prostates for the hog and sheep preparations were respectively 9.47 and 9.44 (8 controls, 6.39).

Recent reports (11, 12) describing an andromimetic function of the adrenal cortex (denied by others, 13) in castrated young rats opened to criticism the well-established belief that metakentrin evoked enlargement of the prostate through its stimulating action on the testicular interstitial cells (10). The relevant data obtained by the injection of metakentrin into castrated hypophysectomized immature male rats (table 1) show conclusively that in the absence of the testes no androgen is produced as a result

[2] An implied necessary condition in addition is that metakentrin's action in the hypophysectomized immature female is limited to the ovarian 'interstitial cells.'

[3] Pure sheep ICSH was kindly supplied by Drs. Li, Simpson and Evans.

of such treatment. A few of the anterior prostates in both the treated and untreated castrated hypophysectomized groups weighed slightly more than those of control hypophysectomized animals owing to the spreading of mild surgical edema resulting from castration. Complete prostatic atrophy was confirmed by microscopic examination. Also, it should be mentioned that elsewhere in this report data on hypophysectomized immature female rats treated with 50 μg. of N metakentrin daily for 10 days provide evidence that metakentrin has no adrenocorticotropic action. In related experiments, the androgenicity of adrenal cortical extract and of desoxycorticosterone acetate[4] was tested in hypophysectomized immature male rats. Total doses of cortical extract (Wilson) amounting to 4 to 13 Cartland-Kuizenga units administered over 8 days beginning on the second day postoperatively produced no increase in the weight of the anterior prostate (table 2); histological evidence of prostatic stimulation was

TABLE 2. EFFECT OF CORTICAL EXTRACT AND OF DESOXYCORTICOSTERONE ACETATE ON TESTES AND ANTERIOR LOBE OF PROSTATE GLAND OF HYPOPHYSECTOMIZED IMMATURE MALE RATS

Number of Animals	Material Injected	Total Dose	Days Injected	Average Weight, in mg.	
				Testes	Anterior Prostate
7	Cortical extract, Wilson	4.5 C.K. units[1]	4	113.4	6.0
2	Cortical extract, Wilson	13 C.K. units	7	78.2	4.9
3	Controls, operated 9 days			85.5	5.6
3	Desoxycorticosterone acetate	1 mg.	4	114.6	7.0
4	Desoxycorticosterone acetate	5 mg.	4	120.6	9.6
4	Desoxycorticosterone acetate	10 mg.	4	117.7	8.8
3	Controls, operated 6 days			118.2	7.2

[1] Cartland-Kuizenga units.

Injections were started on the second postoperative day and necropsies made on the day after the last injection.

also entirely lacking. Desoxycorticosterone acetate administered in total doses of 1, 5 or 10 mg. over 4 days produced, with the 5 and 10 mg. doses, a slight but not statistically significant increase in the weight of the anterior prostate; histological evidence of androgenic stimulation was lacking. Testicular atrophy, as judged by organ weight and microscopic appearance of the tubules and interstitial cells, was likewise not delayed by this treatment. Total doses of 50 and 36 mg. of desoxycorticosterone acetate given to castrated immature male rats (14) and guinea pigs (15) respectively have not produced an androgenic action.

Thylakentrin appears to be the pituitary gametogenic hormone. In the hypophysectomized male rat the selective action of this hormone can be demonstrated most conveniently. Thylakentrin-treated animals show an increase in testis weight with an associated atrophy of the sex organs (fig. 5) normally maintained by testicular secretion. Related histological findings show seminiferous tubule stimulation and interstitial cell atrophy (fig. 8).

The testes and anterior prostate weights produced by doses of thylakentrin varying from 2 to 360 μg. of N are shown in graphic form in figure 1. The points on this graph represent a compilation of assay data from six different preparations of thylakentrin (as symbolized on the graph). Minimum testicular weight increases were produced by 2 μg. of hormone N and consistently greater responses were obtained with

[4] Dr. David Klein (The Wilson Laboratories) and Dr. E. Oppenheimer (The Ciba Pharmaceutical Products, Inc.) aided us by providing adrenal cortical extract and desoxycorticosterone acetate.

increasing dosages up to 30 μg. of N. One hundred μg. of N produced only a slightly greater testicular response than 30 μg. and no further increases of testes weight resulted from increasing the dose to 360 μg. of N. It will be noted that even the largest doses of thylakentrin caused no significant change in anterior prostate weight whereas more than a 70 per cent increase in weight followed the injection of 2μg. of metakentrin N.

Effects of Metakentrin and Thylakentrin in Hypophysectomized Female Rats

Effects of the hormones administered separately. The ability of metakentrin to stimulate the interstitial cells of the female gonad is most clearly demonstrated in the hypophysectomized immature female rat. The only observable reaction in the ovaries of such treated animals is the stimulation of the interstitium. The atrophic changes occurring in the interstitial cells of the ovary ('thecal cells') following removal of the

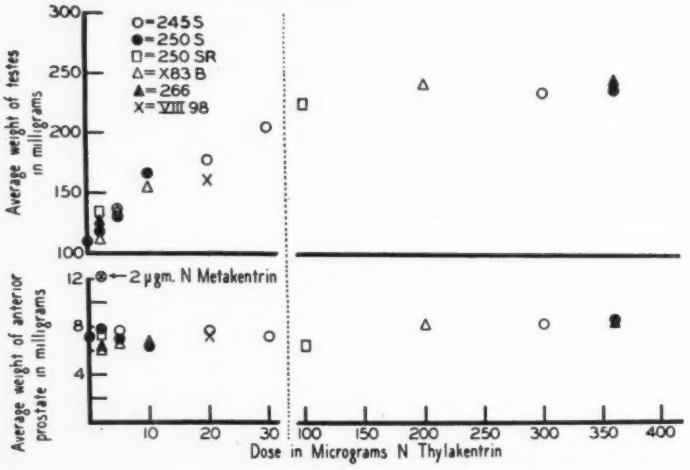


Fig. 1. Effect of various doses of thylakentrin which contained about 20 per cent of inert impurities. Note that testicular hypertrophy is not associated with any significant change in the weight of the anterior prostate. For purposes of comparison the effect of 2 μg. of metakentrin N on the prostate is shown.

These animals were hypophysectomized at 21 days of age (body weight, 35–47 gm.) and the injections were started after a two-day postoperative interval. Each animal received one fourth of the dose in 1 cc. of solution on 4 successive days. Necropsies were made on the day following the last injection.

pituitary gland have been described by Selye, Collip and Thomson (16). Prevention of these changes by treatment begun soon after operation or repair of the atrophic changes by treatment begun several days postoperatively are the criteria of active interstitial cell stimulation. Since we routinely begin our injections on the second postoperative day we essentially maintain or perhaps, in the instance of strong stimulation, produce a hypertrophy of the interstitial cells. It is almost impossible to use normal animals for this test since the interstitial cells of the normal immature rat ovary appear to be already in a strongly stimulated condition.

The weights of several of the endocrine organs of a group of female rats hypophysectomized at 21 days of age and treated with a large dose of metakentrin (50 μg. of N daily) over a period of 10 days are shown in table 3. It will be noted that there were no significant changes in organ weights induced by this treatment. The detection

of hormone activity was made entirely on a histological basis; the evidence of such action was confined to the interstitial cells of the ovary. These were in a maximally stimulated condition (fig. 9, 22). Many of the primordial follicles were converted into what have been sometimes spoken of as pseudo-lutein bodies and the thecal cells in many instances were greatly enlarged. However, new follicles were being formed thus proving that the process of ovogenesis, known to continue long after the removal of the pituitary (17), was not interrupted and possibly not influenced by metakentrin. The occasional presence of microscopic antra in only a few follicles indicated how well the metakentrin had been freed of thylakentrin (fig. 10, 11).

The ovarian interstitial cells appear to have been totally non-functional since the reproductive tract of these rats showed not the slightest evidence of stimulation (fig. 12, 13, 14, and 15) by either of the recognized ovarian hormones. Evans and co-workers (18) observed this fact in the description of their gonadotropic preparation, ICSH.[5]

The interstitial cells of hypophysectomized female rats are much less sensitive to hog-metakentrin stimulation than those of males. Furthermore, hog and sheep metakentrin differ in their ability to stimulate the ovarian interstitial cells. Using the tech-

TABLE 3. EFFECT OF A LARGE DOSE OF METAKENTRIN ON ORGAN WEIGHTS OF HYPOPHYSECTOMIZED IMMATURE FEMALE RATS

Total Dose	Days	Number of Animals	Average Organ Weights, in mg.			
			Ovary	Uterus	Adrenal	Thyroid
μg. N						
500	10	3	7.3	10.6	5.2	4.1
None		3	6.7	11.6	4.8	3.2

nique employed in Evan's laboratory (19) sheep ICSH produced excellent stimulation at a dosage level of 15 μg. of N but none at 1.5 μg. of N, whereas hog metakentrin was inactive at 15 μg. of N. Using our technique (i.e., 21-day-old female rats, a 48-hour postoperative interval and 4 daily subcutaneous injections) sheep ICSH produced a mild reaction at 5 μg. of N and hog metakentrin showed only a bare trace of activity at 15 μg. of N.

The administration of thylakentrin to hypophysectomized immature female rats causes the graafian follicles to grow and mature. These pass through a cycle of maturation and atresia without becoming either luteinized or cystic. When the follicle growth is marked there is no difficulty in ascertaining this fact either by gross or histological methods but when the response is at a minimum the evidence of positive thylakentrin action is not readily identified. This growth of the graafian follicle induced by pituitary action is reckoned from the time of the development of the antrum, its growth prior to this stage being controlled by extra-pituitary factors (17). The ovaries of our control animals killed several days after hypophysectomy do not have antra in the healthy-appearing follicles. An antrum of small size is occasionally seen in degenerating follicles; the latter undoubtedly represent follicles that were large at the time of hypophysectomy. In the treated animals the presence of a definite antrum in several of the healthy follicles may be taken as unequivocal evidence of thylakentrin stimulation. It has not been our experience that one can positively identify follicle stimulation by histological means in ovaries which do not show a slight

[5] In a recent communication, however, in which human chorionic gonadotropin was injected into hypophysectomized adult female rats, Evans, Simpson, Lyons and Turpienen (28) ascribed the estrous vaginal response as "probably due to estrin production by the greatly hypertrophied interstitial cells."

increase in weight. Expediency alone would preclude an attempt to define a unit of thylakentrin purely on a histological basis. The detection of minimum follicle growth by gross inspection, even with the aid of a binocular, cannot be made with any degree of certainty.

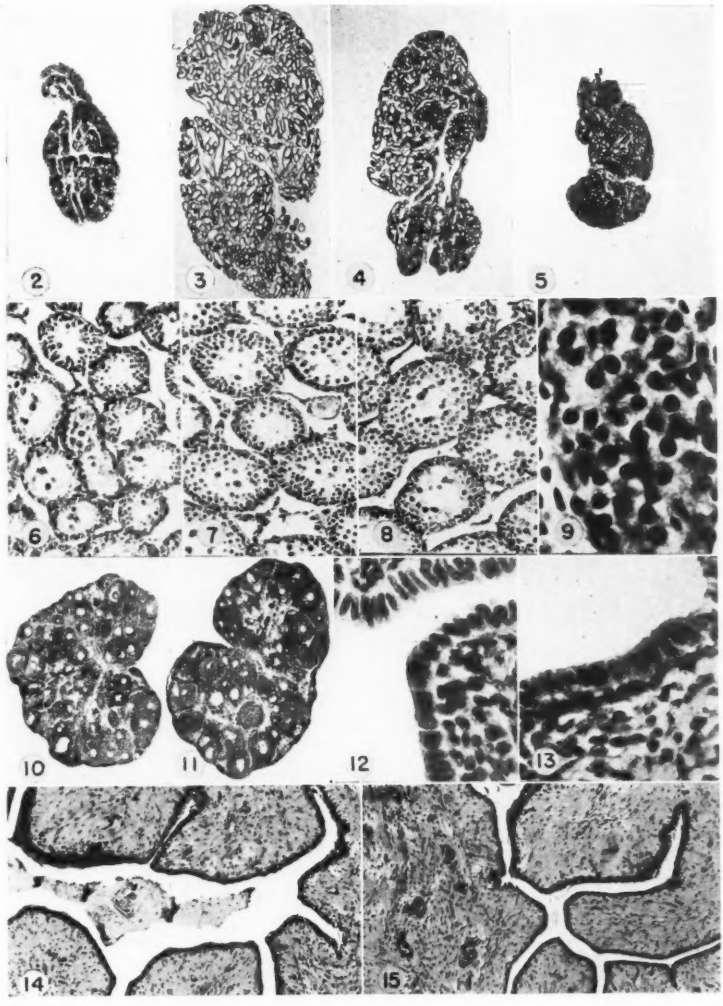
Ten micrograms of N of thylakentrin produced ovaries weighing approximately 12 mg. which represented an increase of 50 per cent over those of untreated operated control animals. Larger doses produced somewhat heavier ovaries within definite and very narrow limits. The ovarian interstitial cells were not stimulated by doses up to 360 μg. of thylakentrin N (fig. 20 shows the effect of 250 μg. of N administered over 10 days). That this hormone does not by itself bring about estrogen secretion (referred to below), has been determined mainly from the weights of the fluid-free uteri, checked occasionally by histological sections of the uterus and vagina. It has been a regular finding that purification of hog thylakentrin especially in the sense of freeing it of interstitial cell-stimulating action resulted in a complete loss of ability to cause estrogen secretion. Fevold (20) found that his FSH preparation produced much better uterine development when administered in combination with LH than when injected alone.

Luteinization and the production of estrogen. Many authors have been reluctant to accept the hypothesis that luteinization of graafian follicles is brought about by a specific hormone. However, in the experiment about to be described we found that pure metakentrin could cause the formation of corpora lutea in immature hypophysectomized rats provided that growth of graafian follicles had been initiated and maintained by thylakentrin. A surprising feature of the combined treatment was the evidence of marked secretion of estrogen, whereas there was no evidence of estrogen secretion after the injection of either hormone alone. This finding was mentioned by us in a previous report (4). Each rat of a group of 33 immature females was hypophysectomized at an age of 21 days. Two days later daily injections of 25 μg. of thylakentrin N were begun and continued for 5 days. On the fifth day 5 animals were autopsied and the remainder divided into two groups each of which continued to receive thylakentrin at the same rate as before; in addition, 25 μg. of metakentrin N were injected daily into one of the two groups. Injections of thylakentrin or of thy-

EXPLANATION OF FIGURES 2 TO 15

Fig. No.	Organ	Magnification	Material Injected	Total Dose μg. N	Days Injected[1]	Animal Number	Weight, in mg.	
							Testes	Ant. prost.
2	Anterior prostate	8	(Hypophysectomized 6 days)			1421	110.6	7.1
3	Anterior prostate	8	Metakentrin	10	4	1934	223.6	37.6
4	Anterior prostate	8	Metakentrin	2	4	1954	148.2	11.9
5	Anterior prostate	8	Thylakentrin	30	4	1515	182.4	5.9
6	Testes	110	(Hypophysectomized 6 days)			1422	101.8	7.9
7	Testes	8	Metakentrin	10	4	1937	212.0	33.8
8	Testes	110	Thylakentrin	360	4	1670	266.0	8.1
							Ovaries	*Uterus*
9	Ovary[2]	500	Metakentrin	500	10	1983	7.3	10.8
10	Ovary	16	Metakentrin	500	10	1983	7.3	10.8
11	Ovary	16	(Hypophysectomized 12 days)			1950	7.4	13.0
12	Uterus	500	Metakentrin	500	10	1983	7.3	10.8
13	Uterus	500	(Hypophysectomized 12 days)			1950	7.4	13.0
14	Vagina	80	Metakentrin	500	10	1983	7.3	10.8
15	Vagina	80	(Hypophysectomized 12 days)			1987	5.7	12.2

[1] All animals were killed on the day after the last injection.
[2] Interstitial cells

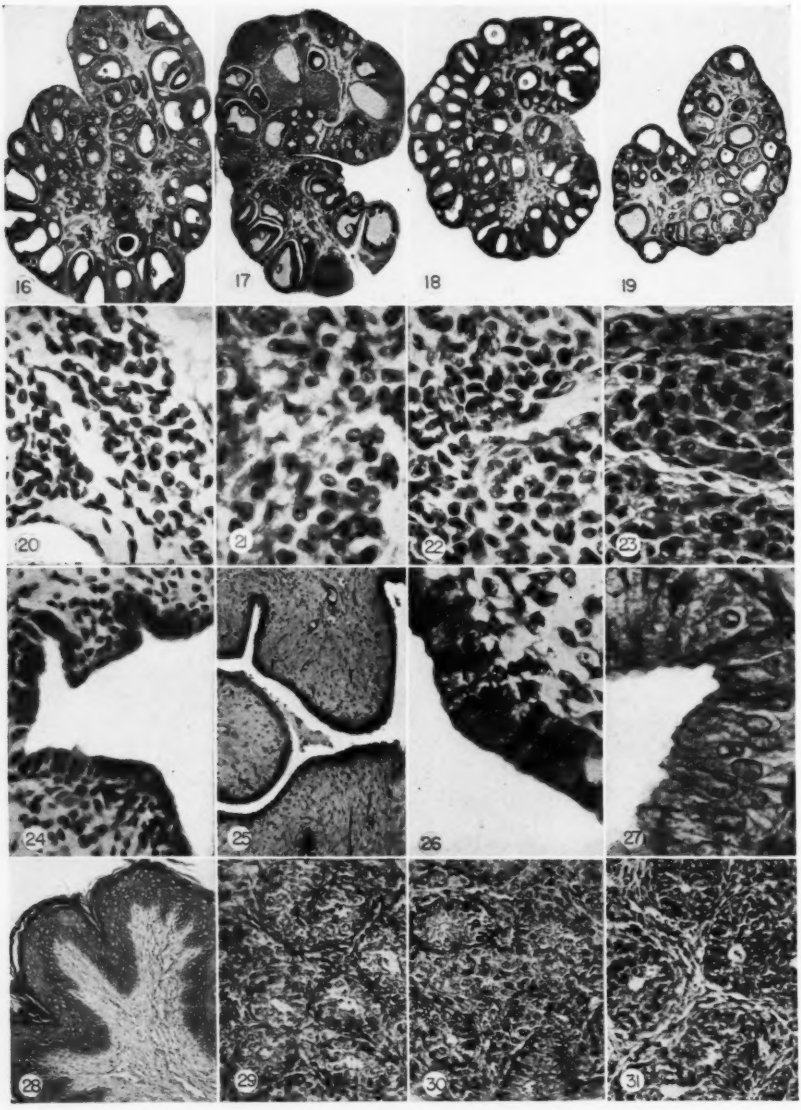


Figs. 2–15

lakentrin and metakentrin were continued 5 days longer. On *days* 8, 9 and *10* a few animals were killed in each group and on the eleventh day, 24 hours after the last injection, the remaining animals in each group, together with uninjected hypophysectomized controls, were autopsied. The ovaries and uteri were weighed and these together with the vaginas were sectioned for histological study.

The ovaries which were obtained on *day* 5 showed an increase in weight over those of untreated hypophysectomized control animals[6] and this was due entirely to

[6] Control rats killed on the fifth day were not included in this experiment. The average weight of the ovaries of 28 control rats autopsied under identical conditions was 8.6 mg.



Figs. 16–31

the growth of graafian follicles as determined by the microscopic examination of serial sections of each gonad. The average weights of the ovaries and uteri of all the groups of animals killed on stated days throughout the remainder of the experimental period are shown in table 4. The growth of the ovaries of animals receiving metakentrin in addition to thylakentrin was clearly potentiated over that produced by thylakentrin alone for any given period of treatment. Our principal concern, however, was focused

TABLE 4. EFFECTS OF THYLAKENTRIN ALONE AND IN COMBINATION WITH METAKENTRIN ON OVARIES AND UTERUS OF THE HYPOPHYSECTOMIZED IMMATURE FEMALE RAT

Thylakentrin	Metakentrin	Number of Rats	Average Ovarian Weight	Ovaries Showing			Average Uterine Weight
				Foll. stim.	Corpora lutea	I.C. stim.	
days[1]	days[1]		mg.				mg.
4	0	5	16.3	5	0	0	15.7
7	0	2	17.9	2	0	0	14.1
7	2	2	23.4	2	0	2	49.5
8	0	3	18.2	3	0	0	16.7
8	3	3	29.4	3	0	3	61.1
9	0	2	14.0	2	0	0	12.5
9	4	4	26.2	4	1	4	78.2
10	0	6	15.1	6	0	0	12.9
10	5	6	22.8	6	5	6	101.4
Hypophysectomized Controls		6	6.7	0	0	0	12.4

Dosages: purified thylakentrin, 25 μg. of N daily; pure metakentrin, 25 μg. of N daily.
[1] Days of treatment are shown in this column. Necropsies were performed 24 hours after last injection.

on the production of corpora lutea. After 48 to 72 hours of the combined treatment there was no sign of luteinization in any of 5 animals (fig. 16); after 96 hours, 1 of 4 animals killed showed several corpora lutea; after 120 hours these bodies were present in 5 of 6 animals (fig. 17). None of the thylakentrin-treated control animals showed the slightest evidence of luteinization at any time (fig. 18, 19) and this again was borne out by examination of serial sections of the gonads.

EXPLANATION OF FIGURES 16 TO 31

Fig. No.	Organ	Magnification	Material Injected	Total Dose μg. N	Days Injected[1]	Animal Number	Weight in mg.	
							Ovaries	Uterus
16	Ovary	13	Thylakentrin	175	7	2002	31.2	64.0
			Metakentrin	50	last 2			
17	Ovary	13	Thylakentrin	250	10	1943	27.9	107.6
			Metakentrin	125	last 5			
18	Ovary	13	Thylakentrin	175	7	2000	16.0	14.3
19	Ovary	425	Thylakentrin	250	10	1933	13.7	11.1
20	Ovary[2]	425	Thylakentrin	250	10	1933	13.7	13.6
21	Ovary[2]	425	Thylakentrin	175	7	2002	31.2	64.0
			Metakentrin	50	last 2			
22	Ovary[2]	425	(Hypophysectomized 12 days)			1950	7.4	13.0
23	Ovary[2]	425	Thylakentrin	250	10	1942	21.3	62.2
			Metakentrin	125	last 5			
24	Uterus	425	Thylakentrin	250	10	1935	13.6	11.1
25	Vagina	65	Thylakentrin	250	10	1936	14.6	12.2
26	Uterus	425	Thylakentrin	175	7	2002	31.2	64.0
			Metakentrin	50	last 2			
27	Uterus	425	Thylakentrin	250	10	1943	27.9	107.6
			Metakentrin	125	last 5			
28	Vagina	425	Thylakentrin	250	10	1943	27.9	107.6
			Metakentrin	125	last 5			
29	Pigeon[3] testis		Untreated control			2021	29.5	*Testes*
30	Pigeon testis		Metakentrin	500	6	2015	41.2	
31	Pigeon testis		Thylakentrin	80	6	2019	62.0	

[1] All animals were killed on the day after the last injection.
[2] Interstitial cells.
[3] Figures 29 to 31 all have the same magnification.

The interstitial cells underwent complete atrophy in all of the animals which received only thylakentrin (fig. 20). The initiation of metakentrin treatment brought about rapid and complete repair of these cells in every instance. At 48 hours definite reparative changes such as increased volume of cytoplasm and decreased nuclear chromophilia were evident (fig. 21, 22). With longer treatment these cells reached a state of extreme stimulation (fig. 23). It should be noted that although luteinization and interstitial cell stimulation are produced under proper conditions by the same stimulus, the effect on the interstitial cells can be recognized much sooner than the luteinizing response.

The uterus and vagina, in reflecting the estrogen output of the ovaries, displayed a most striking behavior. Consistent with our previous 5-day experiments (2, 3) in which highly purified thylakentrin had not caused uterine enlargement, these uteri showed no significant increase in weight even after 10 days of treatment in spite of the fact that the ovaries during all of this time contained maturing follicles, many with large antra, healthy-appearing granulosa, and normal ova. The uteri obtained from the thylakentrin-metakentrin series showed a consistent and progressive increase in weight; within 48 hours the uteri freed of fluid had increased in weight by approximately 350 per cent! All of the uteri taken after the third day of combined treatment were greatly distended with fluid similar to those in the fully developed estrous condition.

The histological preparations of the uterine and vaginal epithelium (fig. 13, 15, 24, 25) strikingly confirmed the fact that no estrogen was secreted during thylakentrin treatment whereas the addition of metakentrin called forth an immediate outpouring of estrogen (fig. 26, 27, 28).

Effect of metakentrin on luteal function. The weight of recent evidence (21–26) favors the view that the stimulus causing the formation of the corpus luteum is not responsible for its continued function. The present experiments were designed to test the ability of metakentrin to sustain luteal function after the latter had been established by natural processes. Adult female rats in estrus were rendered pseudopregnant by electrical stimulation of the cervix. Four days later the uteri were also subjected to electrical stimulation by means of a long slender unipolar electrode passed into the uterine cavities through the cervical canals. This procedure produces without fail a massive deciduoma in pseudopregnant rats after 4 days. By removing the pituitary gland on the day of uterine stimulation and substituting injected metakentrin for the luteotropic influence of the pituitary a positive decidual reaction would supply proof of continued luteal function. Five rats (and their appropriate controls) prepared in this manner were injected with 25 μg. of N metakentrin daily for 4 days. The uteri at autopsy were all threadlike and microscopic examination showed no evidence of a positive decidual reaction whereas large deciduomata were found in the control rats which were not hypophysectomized and had received no metakentrin.

Effect of metakentrin on the corpora lutea persisting in hypophysectomized adult female rats. It has been reported that the pituitary luteinizing fraction, admittedly impure but essentially free of follicle-stimulating effects, caused the involution of these persisting corpora (26). Some confirmatory evidence has been obtained (27, 28). The combination of the LH and FSH fractions of Fevold produced the more rapid involution. It also can be readily demonstrated that crude extracts of the pituitary destroy these bodies when treatment is started at a considerable time after hypophysectomy. Certainly all attempts to re-initiate progesterone secretion in these corpora have met with complete failure (24, 26, 28). In a single well-controlled experiment in which metakentrin was given in what would appear to be an adequately high dose we found no evidence that the pure luteinizing hormone has a destructive action

on such persisting corpora lutea. A group of 17 adult female rats was pretreated for 4 days with chorionic gonadotropin[7] prior to hypophysectomy. The right ovary of each animal was removed and weighed on the 7th day after hypophysectomy and during the following 10 days 9 of these animals received injections of 25 μg. of metakentrin N daily; the remaining 8 animals served as untreated controls. On the 17th day after hypophysectomy both the treated and control animals were killed, and the left ovaries weighed and fixed. A comparison of the average weights of the ovaries of the treated and untreated groups, removed on the 7th and 17th day after hypophysectomy (table 5) shows that no significant decrease in ovarian weight had occurred as a result of the metakentrin treatment. This indication that no luteal involution had occurred was completely substantiated by gross and histological examination of the ovaries. Numerous corpora lutea were present in similar numbers in the ovaries of all the rats.

TABLE 5. EFFECT OF METAKENTRIN ON THE CORPORA LUTEA OF THE OVARIES OF HYPOPHYSECTOMIZED ADULT FEMALE RATS

Number of Animals	Average Ovarian Weight, in mg.		Corpora Lutea
	Right ovary removed 7 days after hypophysectomy	Left ovary removed at 17 days after hypophysectomy	
8 untreated controls	23.7	20.4	Numerous
9 treated by metakentrin after removal of right ovary	23.2	21.4	Numerous

Twenty-five μg. of hormone N were injected daily for 10 days beginning 7 days postoperatively (all rats pretreated with chorionic gonadotropin for 4 days prior to hypophysectomy).

Does Metakentrin Antagonize Mare Serum Gonadotropin?

The mechanism whereby certain pituitary preparations administered intraperitoneally lessen the gross effectiveness of another gonadotropin injected subcutaneously is not yet clear. However the failure of this phenomena to occur in hypophysectomized rats (29) makes it certain that the antagonistic influence is not exerted exclusively against the hormone injected subcutaneously as has been widely supposed. Fraenkel-Conrat et al (30) have suggested that ICSH acts by antagonizing the FSH produced by the test animal's own pituitary gland. An alternative suggestion is that the antagonizing hormone may exert its effect through an inhibition of the production of endogenous thylakentrin. This phenomenon of antagonism has been variously attributed (31) to the action of a specific pituitary gonadotropin, the antagonist, and to other pituitary factors but, principally, to the luteinizing fraction. Consequently, it was of interest to determine the effectiveness of metakentrin in this regard. Our data are not extensive but they do provide a conclusive answer to the above question. Normal immature 21-day-old female rats received 3 daily intraperitoneal injections of pregnant mare serum extract or the serum extract at the same dose level plus varying amounts of metakentrin. The hormones were combined *in vitro*. At necropsy on the second day after the last injection the ovaries and uteri were removed and weighed. Clear-cut inhibition was produced by metakentrin (table 6) in doses of 25 μg. of N or greater but not by 10 μg. of N. The weights of the uteri were not lessened and the qualitative character of the ovarian response was not altered. For example, corpora lutea were always present.

[7] The chorionic gonadotropin used was Follutein.

TABLE 6. METAKENTRIN REDUCES OVARIAN RESPONSE TO MARE SERUM GONADOTROPIN

Number of Animals	Treatment		Average Weight, in mg.	
	PMS extract mg. total dose	Metakentrin μg. of N, total dose	Ovaries	Empty uterus
7	0.24		121.7	120.8
8	0.24	10	119.2	114.1
8	0.24	100	34.9	121.0
6	0.15		59.7	128.0
6	0.15	25	41.7	104.9
6	0.15	50	37.1	109.4
11	0.15		69.0	103.0
10	0.15	50	33.2	107.0

The hormones were combined *in vitro* and injected intraperitoneally (for 3 days) into 21-day-old female rats. Necropsies were made on the second day after the last injection.

Assay of Metakentrin and Thylakentrin by the Rabbit-Ovulation Test

A variety of stimuli, chemical, electrical, or physiological has been used to bring about ovulation in normal rabbits. Hence, it is clearly impossible to use this test as a qualitative assay for gonadotropins. While we do not mean to detract from the extraordinary importance of this test in the clinic and for certain quantitative purposes we do feel that its value in experimental work on the pituitary gonadotropins is extremely limited.

Preliminary experiments, in which the dosage was not based on body weight, indicated that on the basis of N, metakentrin and thylakentrin were about equally effective in causing ovulation in postpartum does (table 7). Because of the difficulties and delay experienced in obtaining these animals a change was made to isolated does in spontaneous estrus, as determined by the color and edema of the vaginal orifice, with the dosage of hormone N being based on each kilogram of body weight. This data (table 8) showed metakentrin slightly more effective than thylakentrin in producing ovulation. Data on experiments in which the two hormones were combined *in vitro* and injected intravenously are also given. The combination of a minimal dose of thylakentrin (5 μg. of N per kg.), which ovulated only 2 of 5 animals, with a subminimal amount of metakentrin (2 μg. of N per kg.) produced ovulation in 10 of 11 animals whereas this same dose of metakentrin combined with only 2 μg. of thylaken-

TABLE 7. PRELIMINARY RABBIT OVULATION TESTS

Metakentrin	Thylakentrin	Number of Rabbits	
		Injected	Ovulating
μg. N	μg. N		
100		3	3
25		6	4
12.5		1	0
2		1	0
	100	1	1
	82	1	1
	20	2	2
	10	1	0
	2	1	0

Hormones injected separately into postpartum does.

trin N, produced no ovulation in 5 does. A potentiating effect was demonstrated by these combined injections but it does not appear that either hormone can be so greatly activated by this means as the data of Foster et al (32) would lead one to expect.

TABLE 8. ASSAY BASED ON RABBIT OVULATION

Metakentrin	Thylakentrin	Number of Rabbits	Number of Animals Showing	
			Ovulation points	Hemorrhagic follicles only
μg. N/Kg. Swine	μg. N/Kg. Swine			
2		11	0	0
4		5	2	1
	25	4	4	0
	5	10	2	5
2	5	11	10	0
2	2	5	0	1
Sheep				
4		3	3	
2		7	7	
1		8	8	

Hormones injected separately and in combination into does during spontaneous estrus.

A preparation of pure sheep ICSH[3] proved consistently more effective in ovulating does than did hog metakentrin. The sheep ICSH at a dose of 1 μg. of N per kilogram of body weight produced a positive response in all of 8 animals whereas hog metakentrin at a dose 4 times greater had this effect in only 2 of 5 does.

Effect of Metakentrin and Thylakentrin on the Growth of the Gonads of 4 to 5-week-old Immature Male Pigeons

The data from these experiments may be seen in table 9. The birds were injected subcutaneously once daily for 5 days and killed on the day after the last injection. Total doses of 4 and 20 μg. of metakentrin N produced no increase in gonad weight and the high dose of 500 μg. of N produced only a 44 per cent increase. The smallest dose produced no histological changes in the gonads; an equivocal stimulation of the interstitial cells was produced by 20 μg. of N whereas using 500 μg. the hypertrophy

TABLE 9. EFFECT OF GONADOTROPINS ON GROWTH OF TESTES OF IMMATURE MALE PIGEONS (4-5-WEEKS-OLD)

Metakentrin	Thylakentrin	Number of Birds	Average Weight of Testicles
μg. N	μg. N		mg.
0	0	4	25.9
4		2	27.5
20		3	25.3
500		2	37.4
	5	2	38.1
	80	2	56.3

Injections were made subcutaneously once daily for 5 days and necropsies were performed on the sixth day.

of the interstitial cells (fig. 29, 30) was sufficient to account for the increase in gonad weight. The seminiferous tubules were not enlarged. As great an enlargement of the gonads (46 per cent) was produced by 5 μg. of thylakentrin N as by a dose of metakentrin 100 times as great. The thylakentrin treated animals all showed definite stimulation of the tubules and no apparent change in the interstitial cells (fig. 31). Gross evidence of androgen production was lacking in all the birds irrespective of treatment.

SUMMARY

Metakentrin (ICSH) isolated from swine pituitary glands stimulated the testicular interstitial cells of hypophysectomized immature male rats to secrete androgen resulting in growth of the accessory organs. The testicles underwent an increase in weight which was presumed to be due to the beneficial effect of endogenous androgen acting on the seminiferous tubules. Castrated hypophysectomized males similarly treated showed no growth of the prostate. Adrenal cortical extract and desoxycorticosterone acetate had no effect on the prostate gland or the testes of hypophysectomized males. The testes of immature male pigeons were not easily affected by metakentrin but with large doses the interstitial tissue was greatly increased. In hypophysectomized immature female rats metakentrin maintained or repaired the ovarian interstitial cells and given in conjunction with thylakentrin (FSH) brought about the formation of corpora lutea. In adult female rats metakentrin did not have a destructive action on the corpora lutea persisting after hypophysectomy. The secretory activity of the corpora lutea of pseudopregnant rats was not maintained by metakentrin following hypophysectomy. The gonadotropic action of pregnant mare serum was greatly reduced by metakentrin.

Thylakentrin (swine) has a specific action on the seminiferous tubules of hypophysectomized male rats. The effects of graded doses in causing testicular enlargement (with no accompanying growth of the prostate) was determined. In hypophysectomized immature female rats thylakentrin brought about growth of graafian follicles without accompanying luteinization, effected no growth of the uterus or cornification of the vaginal epithelium, and failed to stimulate the ovarian interstitial cells. All of the criteria of estrogenic action rapidly became apparent however when metakentrin was administered in combination with thylakentrin.

On the basis of hormone N the ability of metakentrin (swine) and thylakentrin (swine) to cause ovulation in estrous does did not greatly differ. Combined injections of both hormones showed only a moderate potentiating effect by this test.

A general comparison of swine metakentrin with sheep ICSH showed no qualitative differences but certain quantitative differences were found. By the anterior prostate test in hypophysectomized rats the hog and sheep hormones were about equally effective whereas sheep hormone was more effective than hog in causing ovulation in rabbits or stimulation of the ovarian interstitial cells in hypophysectomized rats.

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GONADOTROPINS OF THE SWINE PITUITARY

II. PREPARATION, AND BIOLOGICAL AND PHYSICO-CHEMICAL CHARACTERIZATION OF A PROTEIN APPARENTLY IDENTICAL WITH METAKENTRIN (ICSH)

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LAST YEAR (1) we reported the isolation of a protein, 'metakentrin' (2), from the hog pituitary gland, which repairs, maintains, and stimulates the interstitial cells of the testes and ovaries and causes the formation of corpora lutea from preformed graafian follicles. This protein was shown to be homogeneous by ultracentrifugation and electrophoretic analysis and also to possess a constant solubility in one solvent. The view that the protein is the hormone could not be taken as proved. For the sake of simplicity, however, this protein has been referred to as a 'hormone' without any qualifications because there are no experimental results known to be in conflict with this view. In this paper we shall describe a simplified procedure for the preparation of the hormone together with experiments consistently supporting the belief that the protein is the hormone rather than an inactive protein on which an extremely active hormone is adsorbed. An example of the electrophoretic differentiation of swine and sheep metakentrin will also be presented.

TYPICAL EXAMPLE OF METHOD OF PREPARATION

Four and one-half kilograms of ground fresh hog pituitary glands were extracted with 22.5 liters of cold 2 per cent NaCl solution in the cold room (4°C.). After vigorous stirring for a few hours, the reddish suspension was kept in the cold room overnight. On the following morning, varying amounts of N/70 HCl were added to a series of tubes containing 5 cc. of the suspension. After centrifugation the pH and the nitrogen content of the supernatant fluids were determined permitting the estimation of the pH at which maximum precipitation occurred. The calculated amount of N/1 HCl was then added to the large batch to lower the pH to within 0.05 unit of the point of maximum precipitation. This range was pH 4.2 to 4.6 in various experiments. The suspension was centrifuged and the residue and precipitate were washed twice with 10 liters of cold 2 per cent NaCl. To the combined supernatant fluid and washings was added solid ammonium sulfate (660 gm. per liter of fluid). The protein-free filtrate was discarded. The filtered protein cake was suspended in a minimum amount of water and dialyzed in the cold until free from sulfate. A black sediment was removed by centrifugation and discarded after it had been washed twice with 150 cc. of water. From the combined supernatant solution and washings (total volume 4000 cc.) a protein fraction was precipitated by adjusting the pH with dilute NaOH. The

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amount of NaOH necessary to effect maximum precipitation was previously determined in small aliquots in a manner similar to that already described. The pH of maximum precipitation in various experiments was 5.1 to 5.5. The protein was removed by centrifugation and washed once with water and discarded. The supernatant fluid and washings (4200 cc.) were combined and made to 0.33 saturation of ammonium sulfate. The suspension was allowed to stand at room temperature overnight. The inert precipitate was removed by centrifugation and washed twice with 0.33 saturation of ammonium sulfate. The combined supernatant solution and washings were made to 0.90 saturation of ammonium sulfate. The precipitate was filtered with suction and was dialyzed against distilled water in the refrigerator. When free from sulfate, the protein solution was again made to 0.33 saturation of ammonium sulfate. A

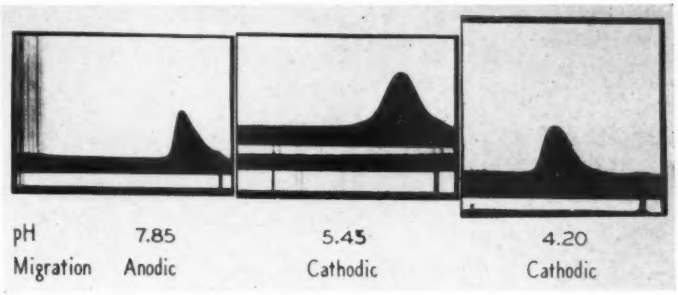


Fig. 1. Electrophoretic patterns of hog metakentrin at different pH.

small amount of precipitate was centrifuged off and discarded after the suspension had stood overnight. The pH of the supernatant fluid was adjusted to 7.3. The suspension was centrifuged. The supernatant solution was found to contain the thyrotropic and follicle-stimulating hormones as well as some metakentrin. The precipitate which contained the main bulk of metakentrin was dissolved in 20 cc. of water and reprecipitated with 10 cc. of saturated ammonium sulfate and with adjustment of pH to about 7.3. These steps were repeated at least seven times. Finally the precipitate was dialyzed until free from sulfate. The yield of the hormone according to this procedure was 0.92 gm. of N from 4.53 kg. of fresh gland. The product appeared as a single component (fig. 1) in electrophoretic analysis of about 1.0 per cent solution at different pH values, with mobilities [(cm./sec.)/(volt/cm.)] of -4.01×10^{-5} at pH = 4.20; -2.45×10^{-5} at pH = 5.45; and 0.52×10^{-5} at pH = 7.85. All experiments were performed at the constant ionic strength of 0.05; only monovalent buffers (acetate and diethylbarbiturate) were employed.

This preparation was also found homogeneous in the ultracentrifuge. A pattern of sedimentation obtained by Svensson's modification of Philpot's method is shown in figure 2. The sedimentation constant was determined in an acetate buffer solution (0.05M) at pH = 4.6 with 1 to 4 per cent NaCl. The mean value brought to a water basis and extrapolated to zero concentration was S°_{20} 6.8×10^{-13}. This figure is higher than the value published previously, but the latter was the experimental value S_{20} uncorrected for the viscosity and density of the solvent as well as for the concentration effect of the protein. If these are taken into account, both figures are in agreement. The diffusion constant as previously mentioned was not determined accurately. Until a better determination has been made, the molecular weight can be tentatively estimated as 100,000.

It should be added, however, that one preparation a few months old, unlike those freshly extracted, exhibited two definite boundaries during sedimentation. In addition to the main component with $S^{\circ}_{20} = 6.8 \times 10^{-13}$, there was a second one with an approximate constant $S^{\circ}_{20} = 2.5 \times 10^{-13}$. The concentration of the smaller unit was roughly 30 per cent that of the larger one. Runs made at zero degree and also at different concentrations in salt and protein did not change the relative amounts of the two units. A separation of the two components was accomplished in a separation cell. The perforated plate supporting the filter paper was located at one-third of the height of the cell from the bottom. A small amount of the slower moving component was obtained free from the faster one. Assays showed that it was biologically active. The limited amount of material, however, did not permit us to make more accurate determinations of its biological activity.

All biological assays were performed in hypophysectomized immature male and

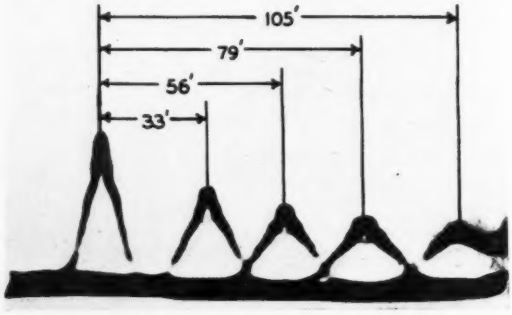


Fig. 2. SEDIMENTATION PATTERNS OF A 3 PER CENT SOLUTION OF HOG METAKENTRIN IN 1 PER CENT NaCl SOLUTION

female rats particularly with regard both to stimulation of the growth of the anterior prostate (3) and testes and to ovarian stimulation limited to interstitial tissue (4, 5).

EVIDENCE THAT THE PROTEIN AND THE HORMONE ARE IDENTICAL

That the hormone metakentrin is a protein is demonstrated by the fact that crystalline proteolytic enzymes such as trypsin or chymotrypsin destroy its biological activity when present in cruder extracts (6). However, crystalline pepsin acting on crude extracts impaired the biological activity of metakentrin only when the mixture of proteins in the extract underwent at least 60 per cent digestion. Although such digests could stimulate interstitial cells, we found that the extent of interstitial cell hypertrophy induced by large doses of peptic digest was definitely reduced, as compared with that by the non-digested control. We have repeated the peptic digestion experiment (table 1) using pure metakentrin instead of the crude extract. It was found that when 88 per cent of the protein had undergone digestion much of the biological activity (75 per cent) of metakentrin was destroyed. Similar experiments using crystalline trypsin were performed, and the results demonstrate that the pure protein is not biologically active after nearly complete digestion by trypsin.

If the evidence, principally in the form outlined in the preceding paragraphs, be accepted regarding the protein-like nature of metakentrin, there remains the important task of demonstrating, if possible, that the pure protein is the hormone. In practice,

molecules of proteins are so complicated in structure that it is extremely difficult to show that a given preparation contains only one molecular species however pure it may appear according to all available tests. Another method of approach is to attempt to separate a more active moiety from the pure protein. Should such an attempt succeed, it would appear that the true hormone is an accidential contaminant of the apparently pure protein. In the remaining part of this report we shall describe typical experiments in which we attempted but failed to detect any fractions more active than the pure protein. Various fractions were tested after electrophoretic migration or ultracentrifugation of the protein or after the protein had been dissolved in a solvent containing Na_2SO_4 and acetate buffer.

TABLE I. EFFECT OF DIGESTION BY CRYSTALLINE PEPSIN AND TRYPSIN ON BIOLOGICAL ACTIVITY OF HOG METAKENTRIN

	Percentage[1] Digestion	Number of Animals	Average weight of prostate after administration of				
			0	2μg. N	5μg. N	20μg. N	50μg. N
Pepsin	88	6				9.7	
Acid Control[2]	0	7		6.8			
Acid Control	0	5			9.8		
Trypsin	60	5					6.3
Alkaline Control[2]	0	4		11.0			
Alkaline Control	0	6			11.7		
Non-Injected Control	—		7.2				

[1] For calculation of percentage digestion, see reference 6.

[2] Acid or alkaline controls were hormone solutions whose pH had been adjusted to those for digestion (pH=4.5 for pepsin and pH=7.4 for trypsin) and incubated at 37°C. for the same length of time as the digestion mixtures.

Fractions obtained after electrolysis. A 2 per cent protein solution was electrolyzed in a Tiselius apparatus and was found to contain only one component. In figure 3, the movement of the protein is shown on the top part of the diagram in which the two

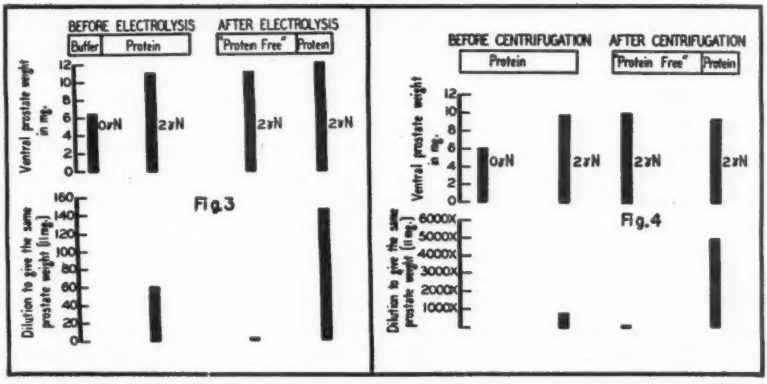


Fig. 3. BIOLOGICAL ASSAY OF PROTEIN SOLUTIONS IN DIFFERENT SECTIONS OF AN ELECTROPHORESIS CELL, AFTER ELECTROLYSIS. Assays were performed in hypophysectomized immature male rats (3). Five rats were used for each group. Fig. 4. BIOLOGICAL ASSAY OF PROTEIN SOLUTIONS IN DIFFERENT SECTIONS OF AN ULTRACENTRIFUGE CELL, AFTER CENTRIFUGATION. Assays were performed in hypophysectomized immature male rats (3). Five rats were used for each group.

long horizontal rectangles represent a limb of the Longsworth type of cell, before and after electrolysis. After electrolysis at pH 4.56 for 14,000 seconds, over 90 per cent of the protein had traveled almost the entire length of the cell. The solution ('protein-free') in the section of the cell from which most of the protein had migrated was analyzed for nitrogen and also used for biological assay. The solution ('protein') in the section of the cell where the protein had been concentrated, was treated likewise. If the hormone were not the protein and had a lower electrical mobility than the protein at this pH, then it would be expected that the 'protein free' solution should have relatively higher biological activity per mg. of nitrogen. On the other hand, if the protein is the hormone, the activity of the remaining small percentage of protein that had not migrated completely should have the same activity per unit of nitrogen as either the main fraction or the original material. The experimental results showed no evidence of increase of activity. On the contrary, the protein in both sections of the cell were indistinguishable in biological activity.

EVIDENCE FROM ELECTROPHORESIS EXPERIMENTS THAT SWINE AND SHEEP METAKENTRINS ARE DIFFERENT PROTEINS

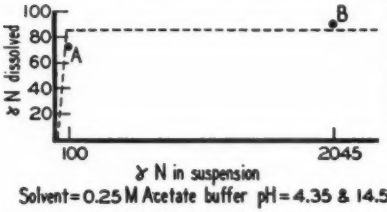
Li, Simpson, and Evans (7) isolated from sheep pituitary glands a protein with biological effects qualitatively like those of the metakentrin we have extracted from hog glands. However, the sheep metakentrin of Li and his colleagues probably is significantly more potent by two of three tests (production of ovulation in rabbit, stimulation of interstitial cells in ovaries but not in testes of hypophysectomized rats) than our hog metakentrin and is notably different with regard to its isoelectric point. The isoelectric point of our swine metakentrin was shown to be at pH 7.45 whereas Li, Simpson, and Evans estimated that the isoelectric point of their sheep metakentrin is at pH 4.6 to 4.8. They mentioned possible reasons for the differences between the two preparations; to us, the most likely reason appears to be the difference in the species source of the protein hormone.

A similar experiment was performed at pH 7.8 instead of 4.56, and similar results were obtained.

Fractions obtained after ultracentrifugation. In one experiment a 2 per cent protein solution containing one per cent sodium chloride was ultracentrifuged. The movement of the protein-buffer boundary is shown at the top of figure 4, where two horizontal rectangles represent the cell before and after centrifugation. The solution contained only one protein component. The 'protein-free' section of the cell, from which most of the protein had been removed by ultracentrifugation, as well as the protein-containing section were separated, analyzed for N, and assayed biologically. The results are shown graphically in figure 4. The 'protein-free' section contained no hypothetical substance with a biological potency higher than that of the protein. Moreover, the biological activity of the protein was unchanged.

Attempted fractionation in Na_2SO_4 solution containing acetate buffer. If the protein together with a second substance such as the hypothetical hormone were dissolved in a suitable solvent, it is probable that the solubilities of the two substances would differ. If the hormone were less soluble than the protein and a suspension of the mixture in slight excess of saturation were made, the insoluble material should be more active than the mixture. On the other hand, if the hormone were the more soluble, the supernatant fluid of a suspension containing a large excess of the mixture should be unquestionably more potent than the mixture. These possibilities have been explored and the results of such an experiment are summarized graphically in figure 5. At point A, 72 of 102 μg. of protein N suspended in each cc. of solvent was dissolved

and assays were performed with the dissolved and undissolved fractions. At point *B*, there was a great excess of protein in suspension (twenty times that of point *A*) and there was an increase of dissolved protein N from 72 to 92 μg. per cc. The dissolved protein N represented 4.5 per cent of the total in suspension at point *B* whereas at



γ N per dose	Average Weight of Ventral Prostate in Mg.		
	Supnt. A	Ppt. A	Supnt. B
0	6.4	6.4	6.4
2	10.7	9.8	11.0
5	10.6	13.6	12.8

Fig. 5. Biological activity of fractions of protein obtained by fractionating pure metakentrin. Assays were performed in hypophysectomized immature male rats (3). Note that the solvent contains less Na_2SO_4 than used previously (1). Eight to ten rats were used for each group.

point *A* 71 per cent of the total suspended was dissolved. Biological assay of dissolved and undissolved protein at *A* and of dissolved protein at *B* revealed no significant differences in activity. Therefore, the data of this experiment also furnish no evidence of the presence of a more active 'hormone' contaminant.

We have compared the electrophoretic behavior of our swine metakentrin with that of a highly purified sheep metakentrin which Dr. H. Jensen kindly furnished to

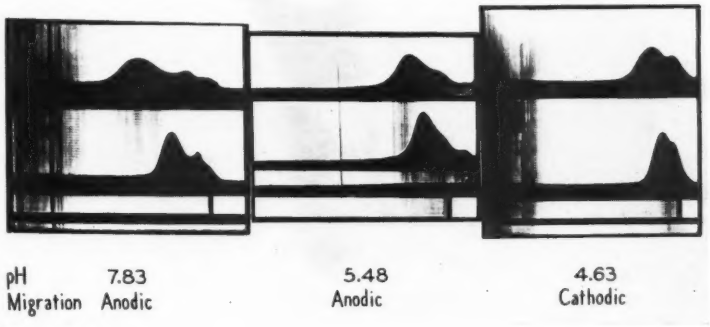


Fig. 6. Electrophoretic patterns of purified metakentrin preparation from sheep pituitary glands.

us. Electrophoretic patterns of Dr. Jensen's preparation are shown in figure 6. By separating as far as possible the two components evident at pH 4.63, we were able to show that the biological activity was associated with the faster moving (larger) component. Since both components moved cathodically at pH 4.63, and anodically at pH 5.48, the isoelectric point of the sheep metakentrin here used can be estimated to be about pH 4.8 to 5.0. This value is not far from the estimate of pH 4.6 to 4.8 published by Li, Simpson and Evans. Thus we have obtained further confirmation of previous evidence that hog metakentrin and sheep metakentrin are different proteins.

In the communication following this (8), the immunological specificity of hog

metakentrin is demonstrated. Again there is full support of the hypothesis that the two protein hormones from different sources, although qualitatively alike in their biological action, are different in chemical structure.

DISCUSSION

The investigations reported here and elsewhere show that a homogeneous protein behaving like the hormone metakentrin (ICSH) can be isolated from hog pituitary glands. The important question to which we attempted to secure an answer is: is the hormonal activity a property of the protein itself or is it the property of another molecular species associated with the protein? Because our present physico-chemical tests for the purity of a protein either by electrophoretic analysis, or by ultracentrifugation or by solubility measurement do not permit the detection of small amounts of impurities, it can always be argued that a minute amount of the active substance is present as a contaminant of the protein. Therefore, we have attempted, although without success, to concentrate a hypothetical, active substance by all the means at our command. Separation and biological testing of fractions obtained in solubility measurements of the protein, after electrophoretic migration of the protein, or after ultracentrifugation of the protein all failed to furnish any evidence of the presence of an impurity more active than the homogeneous protein. We realize that the activity might be located in a unit adsorbed on a protein with a molecular weight of about 100,000. The force of adsorption could be strong enough to prevent splitting during electrophoresis or centrifugation.

It appears that a different highly active unadsorbed contaminant is not responsible for the biological effects. A view to the contrary would require all the following assumptions which as a group appear very improbable; *a*), the hypothetical active contaminant would have the same electrophoretic mobility as the protein at a pH ranging from 4.53 to 7.83; *b*), it would have the same sedimentation constant as the protein when subjected to ultracentrifugation; and *c*), its solubility in acetate-sodium sulfate solvent would be the same as that of the protein.

SUMMARY

A simplified method for the preparation of pure metakentrin from the hog pituitary gland has been described. Advantage was taken of the precipitation of the hormone protein at its isoelectric point.

Evidence that the isolated protein is the hormone is based on constant biological activity with reference to fractions separated incident to solubility measurement, electrophoretic analysis and ultracentrifugation.

Metakentrins isolated from swine and sheep pituitary glands differ in their isoelectric point, as shown by electrophoretic analysis.

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GONADOTROPINS OF THE SWINE PITUITARY

III. IMMUNOLOGICAL SPECIFICITY OF SWINE METAKENTRIN

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THE ISOLATION OF a protein believed to be pure metakentrin (ICSH) was described in 1940 (1). In a preceding report (2) the latest method of preparation was described in detail and the results of experiments indicating that hormone activity and the pure protein are inseparable were discussed. Immunological methods offer an additional means of demonstrating the specificity (uniqueness) and purity of the isolated protein. Application of these methods as here described demonstrated that hog metakentrin can readily be distinguished immunologically not only from sheep pituitary extract, sheep metakentrin (ICSH) or beef pituitary extract but also from extracts of hog tissues such as muscle, neural and intermediate lobes of the pituitary and even anterior pituitary extract, purified thylakentrin (FSH).

EXPERIMENTAL PROCEDURE

Preparation of source material. The hormone protein used as antigen was isolated from hog pituitary glands, according to the procedure described in the preceding paper (2). The protein solution sterilized by filtering through a Seitz filter was diluted with sterile 0.9 per cent NaCl solution, so that the final solution contained 2.0 mg. of protein per cc. according to micro-Kjeldahl analysis. This stock solution was divided into two parts. To one part was added an equal volume of one per cent alum solution; the alum was neutralized by N NaOH to an extent just permitting solution of $Al(OH)_3$. To the other portion of stock hormone solution was added an equal volume of 0.9 per cent NaCl solution. The final protein concentration of both solutions was 1 mg. per cc.

Immunization of rabbits. The rabbits were divided into two groups of three each.[1] To *group A* was given the alum-hormone protein complex. To *group B* was given the hormone protein but with no alum added. The animals each received intravenously one cc. of the antigen daily for four successive days. After three days of rest, daily injections were again given for four days. A similar series of injections was repeated for a third time. Animals were bled to death on the sixth day after the last injection. The sera were obtained in the usual manner.

All biological assays were performed in hypophysectomized immature male and female rats particularly with regard both to stimulation of the growth of the anterior prostate and testes (males) and to ovarian stimulation limited to interstitial tissue (3, 4).

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[1] Just before immunization, the two groups of normal adult rabbits were bled and their sera were saved for the serological study to be described later.

RESULTS

To determine the presence of specific antibodies in the immunized rabbit sera, we have performed a series of precipitin reactions using as precipitinogen a preparation of hog metakentrin which was shown to be homogeneous by electrophoretic analysis, ultracentrifugation and solubility test. The results of such experiments are given in table 1. It can clearly be seen that specific precipitins have been produced by the injection of hog metakentrin. Therefore, it can be concluded that this hormone obtained from the hog pituitary is antigenic. Although the number of animals used is too small to permit any definite conclusion, it appears that alum potentiates the antigenic power of the protein as would be expected. To show that the antibody is produced by the stimulus of hog metakentrin and not by contaminating proteins common to hog tissue, we have made extracts containing muscle proteins of hog. Such extracts did not react with the hog metakentrin antisera.

Li, Simpson and Evans (5) have isolated from sheep pituitary glands an electrophoretically homogeneous protein possessing the same gonadotropic activities as hog metakentrin but differing from the latter in its physical and chemical properties.

TABLE 1. PRECIPITIN REACTION OF HOG METAKENTRIN ANTI-SERUM OF RABBITS WITH ITS HOMOLOGOUS ANTIGEN

Group	Rabbit serum	Final dilution of hog metakentrin protein				
		1:2,000	1:10,000	1:50,000	1:250,000	1:1,250,000
A	1	++++	++++	++	+	±
A	2	++++	++++	++	+	±
A	3	++++	++++	++	+	±
B	4	++++	++	+	±	−
B	5	++++	++	+	±	−
B	6	++++	++	+	±	−
Normal rabbit serum		−	−	−	−	−

The latter differences may be due to the species specificity of the hormone protein. Since immunological specificity is ascribed to the chemical structure and configuration of the antigen, it would be expected that the chemical differences between the hormones will be reflected in their immunological reactions. We therefore tested the reactivity of sera from rabbits immunized against hog metakentrin with metakentrin from the pituitary of two other mammals. The results are presented in table 2. The precipitinogens used for tests were extracts from the pituitary glands of hog, sheep and oxen, and were found to contain large amounts of metakentrin by biological assays in hypophysectomized rats. These extracts varied in degree of purity as indicated by the number of components observed after electrophoretic analysis in the Tiselius apparatus. Thus, the 2 per cent NaCl extracts of the glands of the three species of animals contained at least three components, whereas, on purification, *142E* and *T41* had fewer components, and *R*LH and *Li*LH had only one component. Since all three extracts from hog pituitary gland contain the metakentrin, it would be expected that they would react with the anti-sera of rabbits immunized with pure hog metakentrin (table 2). On the other hand all extracts made from sheep or oxen pituitary glands failed to react over a wide range of dilutions. Dr. Li has kindly furnished us with an electrophoretically homogeneous preparation of metakentrin isolated from sheep pituitary glands. This material did not react with hog metakentrin anti-serum

TABLE 2. SPECIES SPECIFICITY OF SERUM OF RABBITS IMMUNIZED AGAINST PURE HOG METAKENTRIN

Preparation	Animal species	No. of Components of different electrical mobilities	Final dilution of protein					
			1:80	1:400	1:2,000	1:10,000	1:50,000	1:250,000
RLH	Hog	1			++++	++++	+++	+
142E	Hog	>3			++++	+++	++	+
2% NaCl	Hog	>3			++++	+++	++	+
LiLH	Sheep	1		−	−	−	−	−
T41	Sheep	2		−	−	−	−	−
2% NaCl	Sheep	>3	−	−	−	−	−	−
2% NaCl	Ox	>3	−	−	−	−	−	−

of rabbits. (LiLH table 2). Since the preparative methods of Li, Simpson and Evans (5) are so different from ours, it was thought that possibily sheep and hog metakentrins are originally identical proteins which later lose their identity because of differences in methods of preparation. A study of simple extracts all made by the same method should decide the validity of such a hypothesis. We have therefore tested the specificity of these immune sera with 2 per cent NaCl extracts of pituitary glands of different species of animals. The extraction consisted of suspending the pituitary glands from hog, sheep or oxen in 2 per cent NaCl solution. The pH of the suspension was adjusted to about 5 and the residue was centrifuged off. The supernatant fluid which contained metakentrin according to our biological assay in hypophysectomized male rats as well as other pituitary hormones was neutralized to a pH of 7 and used as a precipitinogen. Even these crude extracts made with identical procedure but from glands of different species of animals failed to react with the hog metakentrin antisera. Therefore, we conclude that hog metakentrin is immunologically species specific.

Since complement fixation is generally considered to be a more sensitive immunological reaction than the precipitin reaction, this test was also employed. The results are given in table 3. Here again, we see that the hog metakentrin antiserum reacts only with its homologous antigen, but not with the metakentrin from other species of animals.

It is well known that pituitary glands may be divided into several lobes, such as the neural, intermediate, and anterior lobes. Hormones of different physiological function are present in the different lobes. It was of interest to learn whether hormones from the different lobes of pituitary glands of same species of animal, i.e.

TABLE 3. COMPLEMENT FIXATION REACTION OF HOG METAKENTRIN ANTI-SERUM OF RABBITS WITH METAKENTRIN FROM DIFFERENT SPECIES OF ANIMALS

Dilution of metakentrin	A, Hog metakentrin				B, Sheep or beef metakentrin				
	Dilution of serum				Dilution of serum				
	1:40	1:80	1:160	1:320	1:20	1:40	1:80	1:160	1:320
1:25,000	0	0	4	4	4	4	4	4	4
1:125,000	0	0	3	4	4	4	4	4	4
1:625,000	0	0	0	4	4	4	4	4	4
1:3,125,000	0	0	0	4	4	4	4	4	4
1:15,625,000	2	0	2	4	4	4	4	4	4

0=no hemolysis; 4=complete hemolysis; 3, 2=less complete hemolysis; Anti-sera and metakentrin protein were not anti-complementary at the dilutions used.

hog, will react with the hog metakentrin anti-serum. For this purpose, posterior lobes (neural and intermediate lobes) of hog pituitary glands were separated from anterior lobes by dissection through the kindness of Dr. R. O. Greep. The posterior lobes were separately extracted with 0.01N H_2SO_4. After centrifugation, the clear supernatant solution was assayed for biological activity. It was found that about 20 micrograms of the total nitrogen in the supernatant fluid were equal to 1 U.S.P. unit of both oxytocic (guinea pig uterus) and pressor (dog) activities. Five hundredths μg. of the total nitrogen were enough to disperse the melanosomes of the hypophysectomized frog. These tests showed that the crude extract was rich in the hormones present in the neural and intermediate lobes. The result of the precipitin reaction given in table 4, shows that except at a relatively high concentration of the extract, there is no detectable precipitin reaction. The slight reaction occurring at high concentration may be due to the presence of diffused metakentrin. Moreover, when the immune serum was tested against purified intermedin which by biological assay was found to be virtually free from hormones of both neural and anterior lobes, it failed to give any precipitin reaction. From these results, it may be concluded that metakentrin is 'lobe-specific' in the sense that its anti-serum does not react immunologically with

TABLE 4. PRECIPITIN REACTION OF HOG METAKENTRIN ANTI-SERUM OF RABBITS WITH OTHER PITUITARY HORMONES EXTRACTED FROM HOG GLANDS

Preparation	Lobe	Final dilution of pituitary hormone protein					
		1:80	1:400	1:2,000	1:10,000	1:50,000	1:250,000
XIV-4-P	Neural and intermediate		±	−	−	−	−
CH-74-A	Intermediate	−	−	−	−	−	
RLH	Pure metakentrin from anterior			++++	++++	+++	+
FSH-*X-83-C*	Thylakentrin from anterior		±	−	−	−	−

hormones from the neural and intermediate lobes of pituitary glands of the same species of animals.

Important hormones of the pituitary gland are found in the anterior lobe. It would be of great interest to determine whether these hormones are immunologically specific. Unfortunately, our present knowledge does not permit us to isolate more than one as a chemical individual. However, we have found (6) that it is possible to obtain by chemical fractionation, an extract which is rich in follicle-stimulating hormone of the anterior lobe, but which contains less than one per cent of metakentrin. The result of the precipitin reaction shows that the hog metakentrin anti-serum failed to react with an extract (FSH-*X-83-C*) which contained at least one other hormone likewise from the anterior lobe of the hog pituitary gland. The slight reaction at high concentration would be expected if approximately 0.1 per cent of metakentrin was present in the follicle-stimulating extract.

DISCUSSION

The hormone metakentrin isolated as a homogeneous protein stimulated in rabbits the production of specific antibodies demonstrable both by the precipitin and complement fixation reactions. Inasmuch as the protein antigen was free from inert protein,

it seems justifiable to conclude that the antibody induced was specifically stimulated by the hormone.

Since the hog metakentrin anti-serum does not react with metakentrin (extracted by an identical procedure or isolated as a single electrophoretic component) from sheep or beef pituitary glands, it is immunologically species-specific, although these hormones are qualitatively similar in their biological action. Ten-Broeck (7) reported early in 1934 that the immunological reactions (anaphylactic shock) of various trypsins are similar to those just described for metakentrin. His results showed that trypsin from swine and cattle, and chymotrypsin and chymotrypsinogen from cattle all act as distinct antigens.

In contrast to the above specificity, Bischoff and Lyons (8) found that their purified lactogenic preparations made from pituitaries of oxen and sheep were antigenically indistinguishable by the usual immunological tests. Whether such an identity represents the property of this particular protein hormone or that of contaminating protein cannot be decided from available data.

It is interesting to note in table 4 that hormone extracts prepared from the neural, intermediate and anterior lobes of hog pituitary fail to react with hog metakentrin anti-serum. Thus both lobe and hormone specificity have been demonstrated immunologically in confirmation of biological findings.

SUMMARY AND CONCLUSIONS

Metakentrin (ICSH) isolated in the pure state from the hog pituitary gland is antigenic.

Its anti-serum does not react with the similar hormone or extract containing that hormone if these have been isolated from other species of animals, e.g. sheep or ox. Therefore, hog metakentrin is species specific.

Its anti-serum does not react with other hormones from different or even the same lobes of the pituitary gland of the same species of animal. Therefore hog metakentrin is not only 'lobe specific' but also 'hormone specific.'

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INCREASE IN GONADOTROPIC CONTENT OF PITUITARY GLANDS OF FEMALE RATS TREATED WITH ANTIGONADOTROPIC SERUM[1]

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SINCE THE ANTIHORMONE CONCEPT was first presented by Collip and his co-workers (1) many papers have appeared corroborating their report of the inhibitory action of antihormone upon simultaneously administered gonadotropins. Further work with a specific antigonadotropic serum has demonstrated its power to inhibit not only exogenous gonadotropic extracts but also the secretion of an animal's own pituitary gland (2–6). Recent experiments by Severinghaus and Thompson (7, 8) and by us (9) have shown that in addition to the inhibitory effect on gonadal growth, changes can be produced in the cell types of the anterior pituitary gland by antihormone treatment. These changes which consist mainly of an increase in number and size of the basophiles and decrease in number of chromophobes are like those seen after castration. In addition to the histological effects, we have shown in other experiments (10. 11) that there is a corresponding increase in secretion of gonadotropic hormone. The hypersecretion was demonstrated by precocious gonadal development occurring in immature female rats after discontinuing the antihormone treatment and by ovarian hypertrophy found in littermate females in parabiosis with antihormone-pretreated males or females. It does not necessarily follow, however, from these experiments that the gonadotropic content of the pituitary gland of animals injected with antihormone is likewise increased since no determinations of gonadotropic activity were made. Therefore, it seemed important to ascertain the gonadotropic content of the pituitary gland of such animals so that a more complete understanding could be obtained of the action of antigonadotropic sera on the gonads and pituitary gland.

The object of this report is to describe the results obtained by assaying the pituitary glands of immature and mature female rats treated for at least 54 days with antigonadotropic sera. In addition, experimental evidence will be presented to demonstrate the rôle of the ovary in controlling the secretion and the production of the gonadotropic complex by the pituitary gland.

PROCEDURE

Immature female rats were given subcutaneous injections of antigonadotropic serum from the first through the sixty-second day of life and the gonadotropic content

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of the pituitary glands was determined at the end of the treatment. The serum was prepared from blood obtained by cardiac puncture from rabbits which had been injected daily for a period of 2 months or longer with a supercentrifuged aqueous extract of whole dried sheep pituitary glands. The serum was non-specific in nature and was capable of inhibiting the gonad-stimulating action of extracts of sheep pituitary glands, pregnant mare serum, and pregnancy urine and aqueous suspensions of dried human and rat pituitary glands. The rats were injected with 0.05 cc. of serum per day from the 1st through the 5th day of life and 0.1 cc. per day from the 6th through the 10th day. This dose was increased to 0.2 cc. per day from the 11th through the 15th day and then to 0.4 cc. from the 16th through the 20th day of life. One-half cc. of serum per day was administered from the 21st through the 62nd day of life. Each animal received a total dose of 24.75 cc. of antigonadotropic serum. The experimental and littermate control rats were autopsied the day following the last injection. The ovaries and the pituitary glands of the two groups of rats were removed at the time of autopsy and weighed to the nearest milligram. The degree of uterine stimulation and the presence of corpora lutea and follicles in the ovary was also recorded. The gonadotropic content of the fresh pituitary glands of both the experimental and control rats was determined by injecting an aqueous suspension of the glands into 21-day-old female rats. Nine injections of 0.5 cc. each were made over a period of 4.5 days on the basis of two per day. The suspension was diluted so that each assay animal received 15 mg. of fresh gland.

A second experiment was planned so that a comparison could be made between the gonadotropic content of the pituitary glands of mature female rats given antihormone and those castrated for a time equal to that of antihormone treatment. Thus, adult female rats of approximately 200 days of age and 200 $\pm$ 15 gm. in weight received 0.5 cc. of antigonadotropic serum per day or a total dose of 27 cc. during 54 days. Rats of the same age and weight as the experimental females were selected for the control groups. Of these, one group was castrated on the first day of the experiment, i.e., at commencement of the antihormone injections. The remaining animals served as intact controls.

Twenty-four of the antihormone-treated animals together with 23 normal and 19 castrate controls were autopsied on the 55th day of the experiment, the day after cessation of serum treatment. The ovaries, uteri and hypophyses of all of the animals were carefully dissected free of extraneous tissue and weighed to the nearest milligram. The presence or absence of corpora lutea and follicles was also noted. The gonadotropic content of the pituitary glands of the three groups of animals was determined by pooling the individual glands from each group and injecting them as an aqueous suspension into 21-day-old female rats. The material was so injected that each assay animal received a total dose of either 15 or 25 mg. of fresh pituitary glands.

Twenty-one additional female rats which were treated with antihormone for 54 days were killed 19 days after cessation of treatment, together with 34 normal and 29 castrate controls. The purpose of this experiment was to determine the gonadotropic content of the pituitary gland of the antihormone-injected rats some time after the injections had been stopped. The weights of the ovary, uterus and pituitary gland were recorded for both the experimental and control animals. The pooled hypophyses from each group were assayed by the method previously described.

A small portion from the same region of the anterior pituitary gland of three or four animals in each of the experimental and control groups of the adult rats was fixed in Bouin's fluid and saved for histological study. Whole pituitary glands of animals treated from the 1st to the 54th day of life with antigonadotropic serum together with

glands from littermate controls were also fixed in Bouin's fluid. All the glands were sectioned at 6μ and stained with a modification of Mallory's trichrome stain (12).

RESULTS AND DISCUSSIONS

The data obtained from the study of the eight litters of immature female rats treated with antisera from the 1st through the 62nd day of life, together with the

TABLE 1. EFFECT OF ANTIGONADOTROPIC SERUM UPON THE GONADS AND GONADOTROPIC CONTENT OF THE PITUITARIES OF IMMATURE FEMALE RATS

Litter Number	Number of Rats	Treatment	Average Ovarian Weight	Pituitary Assay[1]		
				Donors	Number of recipients	Average ovarian weight
			mg.			mg.
1	3	Antihormone[2]	17	Antihormone	1	37
	3	Control	30	Control	1	10
2	3	Antihormone	28			
	3	Control	44			
				Antihormone	2	126
3	3	Antihormone	22	Control	2	19
	4	Control	33			
4	3	Antihormone	11			
	3	Control	44			
5	3	Antihormone	11			
	3	Control	37			
6	3	Antihormone	12	Antihormone	7	64
	3	Control	30	Control	6	13
7	3	Antihormone	17			
	3	Control	36			
8	3	Antihormone	19			
	3	Control	42			
Total, 8 litters	24	Antihormone	15	Antihormone	10	75
	25	Control	36	Control	9	14

[1] Fifteen mg. of fresh hypophyses administered to all rats.
[2] Rats treated from the 1st through the 62nd day of life with sheep antigonadotropic rabbit serum.

data of untreated littermate controls are presented in table 1. These data show that the average ovarian weight of the animals injected with antihormone is less than half that of the littermate controls. The results of the pituitary assay, also presented in table 1, show the hypophyses of the rats treated with antihormone to be more potent than those of the control animals. The pituitary glands of the injected animals were characterized by a condition of extreme basophilism similar to that seen after castration. The numerous basophiles were large and vacuolated in appearance. The control glands exhibited a normal cytological picture.

The autopsy data and the results of the pituitary assay of the antihormone-treated, castrated and normal adult female rats are given in table 2. The weights of the ovaries and the uteri of the animals injected with antihormone were significantly less than those of the control rats. Assays of the hypophyses of the adult female rats on the two doses of 15 and 25 mg. of fresh glands indicate that there was no signifi-

cant difference between the gonadotropic content of the pituitary glands of the animals treated with antihormone and that of the castrate rats. A significant difference, however, did exist between the gonadotropic content of the pituitary glands of the antisera-injected and castrate animals and that of normal females.

The differentially stained slides of the fragments of the pituitary glands of the castrate animals and those treated with antihormone showed that in both cases there was an increase in the size and number of the basophiles over those observed in the glands of the normal control rats.

It was shown in previously published reports (9, 10, 11) that after stopping the antihormone treatment of immature female rats there is an increase in the secretion of

TABLE 2. EFFECT OF ANTIGONADOTROPIC SERUM UPON THE GONADS AND GONADOTROPIC CONTENT OF THE PITUITARIES OF ADULT FEMALE RATS

Treatment	Number of Rats	Average Ovarian Weight	Average Uterine Weight	Pituitary Assay		
				Dose	No. of rats	Average ovarian weight
		mg.	*mg.*	*mg.*		*mg.*
Antisera for 54 days	28	23	318	15	8	61
				25	7	109
Castrated for 54 days	19		139	15	7	75
				25	4	132
Control A	23	48	507	15	10	11
				25	4	13
Antisera for 54 days, autopsied 19 days after last injection	21	54	465	15	11	11
				24	4	21
Castrated for 73 days	29		123	15	15	94
				25	4	126
Control B[1]	34	49	489	15	21	13
				25	4	15

[1] Killed 19 days after *control A*.

the gonadotropic hormone. The results given in the present paper demonstrate that immediately after cessation of antihormone injections the quantity of gonadotropic hormone in the pituitary gland is like that found in the castrate rat. These data indicate that the inhibitory action of the antihormone does not take place in the pituitary gland itself but in the blood after the hormone is secreted.

Further evidence for the increase in gonadotropic activity of the pituitary glands of animals injected with antigonadotropic sera is presented in table 2. These data are concerned with the rats which received antisera for 54 days but were killed 19 days after the last injection. The ovarian weights of these animals exceeded slightly those of normal controls which shows that there was considerable growth of the atrophied ovary after administration of antihormone had been stopped. Accompanying this growth there was an increase in the amount of gonadal hormones as indicated by the enlarged uteri. Assay of the pituitary glands of these animals showed them to be comparable in potency to the normal gland. Microscopic examination of the fragments of the anterior pituitary glands demonstrated that they were similar to normal glands. The increase in basophiles noted immediately after antigonadotropic treatment was not found.

The explanation of the increase in gonadotropic content of the pituitary gland during antihormone administration and decrease 19 days after treatment is based on the concept that the antisera neutralizes the gonadotropic secretion of the pituitary gland during the period of injection. Therefore, the size of the gonads is not maintained and gonadal hormone production is greatly decreased. As a consequence castration-like changes occur in the anterior pituitary gland. Accompanying these changes there is an increase in gonadotropic hormone in both the pituitary gland and the blood (13), which is found immediately after discontinuing the antihormone injections. After withdrawal of the antihormone, ovarian stimulation occurs and gonadal hormones are released and act on the hypophysis. Consequently the increased amount of gonadotropic hormone in the pituitary gland during antihormone treatment is soon depleted and further hyperproduction is prevented. These experiments provide additional information concerning pituitary-gonad interaction and demonstrate the value of antigonadotropic substances in studying this relationship.

SUMMARY

The gonadotropic potency of the hypophyses of immature and adult female rats treated for 63 and 54 days, respectively, with antigonadotropic serum was like that of castrated rats and greatly exceeded the gonad-stimulating power of the pituitary glands of normal rats. The gonads of the animals treated with antihormone were less than half the weight of those of the normal control females. The gonads of the adult female rats killed 19 days after cessation of antihormone injections had entirely recovered from the atrophic state found immediately after discontinuing the anti-gonadotropic treatment. The gonadotropic content of the pituitary gland of these animals was the same as that of the normal control rats. An increase in size and number of the basophiles was noted in the animals killed immediately after the last injection of antihormone. This basophilism was absent in animals autopsied 19 days after the last antihormone injection. The mechanism whereby an increase in gonadotropic content of the pituitary gland follows treatment with antihormone is discussed.

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OVARIAN WEIGHT INCREASE AS AN OBJECTIVE MEASURE FOR ASSAY OF PROLAN

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JUDGING BY THE NUMBER of papers on the assay of prolan, present methods have not proven entirely satisfactory. A number of workers have abandoned the assay based on ovarian hypertrophy, which is specific, for the non-specific assay based on uterine hypertrophy in which the changes in weight are of greater absolute magnitude. In an effort to evolve a more suitable assay procedure, the increase in ovarian weight produced by prolan administration is compared in this study under various conditions designed to produce a greater response in absolute weight. Data reported in the literature are compared and analyzed as to the relative accuracy of the uterine and ovarian weight procedures.

Errors in published ovarian assay procedures. Bischoff and Long (1), D'Amour and D'Amour (2), Chou and Liu (3), and Sealy and Sondern (4) present data for the effect of doubling the dose of prolan upon ovarian hypertrophy in approximately the same ovarian weight range. Respectively, the increases are 18 to 24, 16.2 to 23.6, 18.6 to 26.0, and 15.0 to 24.7 mg. Bischoff and Long gave one dose per day for 4 days, D'Amour and D'Amour gave one dose per day for 3 days, Chou and Liu gave three doses per day for 4 days, and Sealy and Sondern gave two doses per day for 3 days. In all cases except the first, autopsy was performed the 5th day after initial dosage. D'Amour and D'Amour give no measure of variability. Using ten rats per assay, the data of Bischoff and Long and of Chou and Liu indicate that the assay would deviate not more than 30 per cent, 95 per cent of the time. Using nine rats, the data of Sealy and Sondern indicate the deviation of 15 to 20 per cent for the same probability. In a later paper (5), D'Amour and D'Amour using the same technique described in their early paper found that doubling the dose increased ovarian weight from 14.6 to 16.3 mg. In the original paper from this laboratory (1) in which the rats were autopsied the 6th day after initial dosing and in a later paper (6) in which autopsy was performed the 5th day, ovarian hypertrophy was extended by doubling the dose required to produce ovaries weighing 25 mg. In the latter case this was accompanied by an increase of 9 mg. in ovarian weight, with a deviation which indicated that the result would fall within 25 per cent of the correct value 95 per cent of the time in groups of six or seven rats. Chou and Liu obtained an increase of 8.2 mg. in the same ovarian-weight range by a technique which employed 3 doses per day. Employing ten rats per dose level, their data indicate the result would deviate not more than 33 per cent from the correct value 19 of 20 times.

Errors in published uterine assay procedures. Two examples of data obtained in this laboratory are given. Additional data may be found in recent papers (6, 7). In the assay of D'Amour (8), using ten pairs of litter mates, values of 83 ± 7 versus 39 ± 6 mg. were obtained for two dosage levels. According to the curve for this type

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of assay the smaller value represents 64 per cent the potency of the large value. Thirty-six per cent is, therefore, equivalent to 4.8 times the standard deviation of the mean, and the accuracy of the assay is within 15 per cent, 95 per cent of the time. In the assay of Delfs (9) using ten pairs of litter mates, values of 70 ± 8 and 53 ± 10 mg. were obtained for two dose levels. According to the curve for this type of assay the smaller value represents 74 per cent the potency of the large value. Twenty-six per cent is, therefore, equivalent to 1.3 times the standard deviation of the mean, and the accuracy of the assay is within 40 per cent, 95 per cent of the time. In the data presented by Delfs the standard deviation of the mean is somewhat less in most cases than that revealed by the above example from our laboratory. The data presented by Chou and Liu (3) confirm our experience. D'Amour and D'Amour give no measures of variability so that the accuracy of the assay with their rat colony is not known. Sealy and Sondern (4) present measures of variability for their uterine assay procedure which indicate that the error is 15 to 20 per cent, using 8 rats per assay.

EXPERIMENTAL PROCEDURE

In *experiment* 1, the test object was the 22-day-old rat (Evans strain). The period of dosage has been extended 1 day over that initially used (1), the total period being 5 days. Administration was twice daily so that the total number of doses was 10. The rats were autopsied 24 hours after the last dose.

In *experiment* 2, the test object was again the 22-day-old rat. Pituitary gonadotropic hormone was administered with the prolan to produce the augmentation phenomenon. The same dose of pituitary hormone, 0.25 mg. per rat, was administered with all dose levels of prolan. In other respects the details of the assay were the same as those previously recorded.

In *experiment* 3, the age of initial dosage has been extended to 30 days. A total of 8 doses in 4 days was administered.

Experiments 4 and 5 were performed with adult Marsh-Buffalo virgin female mice. In *experiment* 4, a total of 10 doses in 5 days was given. In *experiment* 5, the same total amount of hormone was administered once daily in 4 days.

The prolan used was the stable powder employed in this laboratory since 1936 (1). The solution for assay was made up in isotonic salt solution on the day of initial dosage and kept refrigerated. The volume of injected solution was always the same for any of the comparative doses. In the experiments employing rats the comparison is made between litter mates. The mice were pooled in a large container and picked by random sampling. In table 1 are given the number of animals used per level of dosage, the dosage schedule, the age at autopsy, body weight means with standard deviation of the mean, body weight extremes, ovarian weight means with standard deviation of the mean, ovarian weight extremes, and dose level per animal. In the last column is given the percentage (of the highest dose) within which the assay would be expected to fall 19 of 20 times, on the basis of the variation in response obtained (standard deviation of the mean). In this calculation Sealy and Sondern (4) eliminate any value which exceeds $\sqrt{1/2 \Sigma d^2}$, and we have followed the same procedure.

RESULTS

The results of *experiments* 1, 4, and 5 are more satisfactory than those of *experiments* 2 and 3 in that the experimental error reaches 30 per cent in the latter. In *experiment* 2, this occurs at the higher dosage level. The augmentation of prolan with pituitary hormone in *experiment* 2 does not increase the accuracy of the assay. Since fewer rats were used in *experiment* 3 than in *experiment* 1, the accuracy of the assay

on the basis of rats used is as accurate as that of *experiment* 1. *Experiments* 4 and 5, differing in technique only in the number of doses given, illustrate that accuracy is increased by divided dosage. *Experiments* 1 and 4 reveal a minimum of experimental error throughout the range studied. Any of the methods are satisfactory in establishing the loss of 50 per cent or more of activity in a chemical reaction study with a high degree of probability, the accuracy increasing with increasing inactivation.

DISCUSSION

Hamburger and Pedersen-Bjergaard (10) demonstrated that strains of rats vary greatly in their response to the same dose level of prolan. At certain levels both the uterine and ovarian weight responses were 50 per cent lower in one strain than in

TABLE 1. OVARIAN WEIGHT INCREASE AS AN OBJECTIVE MEASURE FOR ASSAY OF PROLAN

Experiment Number	Number of Animals per Test	Dose Schedule: Times per day	Dose Schedule: Number of days	Age at Autopsy, Days	Ovarian and Body Weights at End of Experiment: Body wt., mean	Body wt., gram range	Ovarian wt., mean	Ovarian wt., milligram range	Dose Level Total per Animal, milligram	Range (—) in Per cent in which Result Falls 19 in 20 Times
1, Rats	13	2	5	27	58±1.0	51–64	37.7±1.1	45–32	0.30	17
	13				57±1.0	48–65	28.9±1.0	37–22	0.15	10
	13				56±2.1	45–64	22.7±0.9	27–18	0.075	11
	10				55±2.0	45–59	16.7±0.9	21–12	control	
2, Rats	10	1	4	26	54±1.4	46–60	40.0±2.4	52–32	0.30[1]	30
	10				54±1.3	48–60	30.9±1.3	36–22	0.15[1]	12
	10				53±1.7	43–59	24.0±1.1	31–20	0.075[1]	9
3, Rats	7	2	4	34	69±1.5	62–73	43.5±1.2	48–38	0.30	15
	7				71±2.2	63–77	29.3±1.6	37–23	0.15	29
	7				69±2.5	59–80	25.5±1.3	32–20	0.075	10
	7				72±2.0	67–82	17.0±0.8	21–14	control	
4, Mice	7	2	5	95	23.4±0.4	21.7–24.8	29.7±1.3	32–24	0.30	16
	7				23.3±0.4	21.5–24.5	21.0±0.7	23–18	0.15	12
	7				23.2±0.1	22.5–24.5	14.7±1.3	20–9	0.075	14
	7				21.5±0.6	20.0–24.0	9.9±0.7	12–8	control	
5, Mice	7	1	4	115	22.5±0.6	19.5–24.0	26.9±1.3	30–21.5	0.30	25
	8				22.5±0.1	21.0–24.0	20.4±0.9	23–17	0.15	10
	8				21.8±0.4	20.5–23.0	14.6±0.7	18–12	0.075	13

[1] Augmentation with pituitary hormone

another. These authors also give the relation of the dose (30 times) of prolan which produces a 400 to 500 per cent increase in ovarian weight to the dose which produces a corresponding augmentation in uterine weight. Since measures of variability are not given, their data do not give information as to whether or not the procedures could be used for assay purposes, or whether one is superior to the other. The graphical method of presentation used indicates that increase in ovarian weight is gradual. In this connection it should be noted that if the data for ovaries were presented on a scale of the same magnitude as that used for the uterus and covering only the effective range, the impression of gradual response is greatly lessened. The work of Hamburger and Pedersen-Bjergaard, while it serves as a basis for discussion of later papers on assay of prolan, should not be cited as a condemnation of ovarian weight increase as an objective measure for assay procedure.

Barlow and Sprague (11) present ovarian hypertrophy data for weights below 20 mg. for a large series of animals. Measures of variability are unfortunately not given. The range of dosage studied by Barlow and Sprague is the effective range for uterine hypertrophy and is below the effective range for ovarian hypertrophy. The work of Barlow and Sprague, therefore, does not give information pertinent to the present study.

Some little misunderstanding has arisen concerning the fitness of assay procedure

for some particular purpose. In preparing prolan for clinical use it is imperative that the assay should be in absolute units. In studying the chemical inactivation of prolan, the percentage of activity remaining is sought, it being immaterial what the original activity was in absolute units. Data presented by Sealy and Sondern may be used to illustrate this point. Their data reveal that for 2 of 8 increments of dosage (increasing the dosage from 2 to 3 units and from 5 to 6 units) in their assay by ovarian weight, there was no increase in ovarian weight. Statistical analysis reveals that, using a dose of 8 units, the accuracy of the assay is within 15 to 20 per cent, in 19 of 20 trials. One unit ($\pm 12\%$) falls within this range of error, the number of times it would be expected to fall on the basis of the probability theorem. It should be obvious that in establishing 1 unit with minimum error by the method of Sealy and Sondern, it would be necessary to use approximately 8 units per rat, as these authors do, and the fact that the rat is not sensitive to 1 unit should not condemn the method.

A summary of the results obtained in this paper by five modifications of the ovarian assay procedure in two species of animals and a survey of existing data in the literature, reveal that the ovarian weight procedure can be as satisfactory as the indirect method of uterine assay if adequate dosage and proper division of doses are maintained, without the use of larger series of animals. The feeling, as revealed by private communication with a number of workers, that the ovarian response cannot be used as a reliable estimation of potency, is not borne out by this study. The condemnation, as expressed by others, would appear to be based on: *a*), the use of strains of animals not sensitive to ovarian hypertrophy; *b*), the failure to use statistical analysis; *c*), the failure to use adequate dosage; *d*), the failure to compare litter mates or to use a homogeneous strain of test animals.

SUMMARY

Three graded doses of prolan, each differing from the next lowest by 100 per cent, were administered to immature female rats of the Evans strain and to mature virgin mice of the Marsh-Buffalo strain. In the experiments with 22-day-old rats, one series received ten divided doses in 5 days, and one series received four doses in 4 days augmented with pituitary gonadotropic hormone. In a third experiment, dosage was begun at the age of 30 days. In the experiments with 100-day-old mice a comparison is made between the influence of ten divided doses with four divided doses.

Statistical analyses of the results obtained indicate that ovarian hypertrophy is as satisfactory an objective measure of the activity of prolan as uterine hypertrophy, if the conditions are properly chosen.

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EFFECT OF THE GONADOTROPIC SUBSTANCE OF PREGNANT MARE'S SERUM ON THE BLOOD PLASMA-ASCORBIC ACID OF THE CASTRATE BOVINE[1]

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THE FINDINGS OF Biskind and Glick (1), Ley (2), Phillips et al (3, 4), Winkler and Binder (5) and Israel and Meranze (6) during the past decade have presented evidence which is strongly suggestive of the importance of ascorbic acid in reproduction. Erb and Andrews (7), reported that the subcutaneous injection of equine gonadotropin in the male and female bovine resulted in a decrease of blood plasma-ascorbic acid of 20 to 67 per cent within 24 hours. The recovery of pre-injection ascorbic acid levels required 8 to 10 days in the cow and longer in the bull unless vitamin C was subcutaneously injected. Erb and Andrews suggested that this drop in plasma-ascorbic acid might be responsible for the frequent failure of gonadotropins to produce satisfactory gonad stimulation. Di Cio and Schteingart (8) simultaneously reported that the injection of ascorbic acid in conjunction with gonadotropin caused a greater effect on the genitalia of both the male and female rat than did the gonadotropic hormone alone. Ascorbic acid itself had no effect on the weight of the genitalia.

This study was undertaken to determine whether the gonads have a rôle in the mechanism which is responsible for the drop in blood plasma-ascorbic acid following the injection of equine gonadotropin or whether the gonadotropic hormone acts independently of the gonads.

MATERIALS AND METHODS

Fourteen grade Hereford steers approximately two years of age were made available by the Animal Husbandry Department for this work. These cattle were castrated before they were 6 months of age and had been maintained on a uniform diet of corn silage and timothy and clover hay for the month preceding this study. Although the blood plasma-ascorbic acid of the bovine cannot be influenced by feeding, the maintenance of a uniform ration was considered desirable.

Blood was drawn from the jugular vein into oxalated tubes, placed in a waterbath, and immediately taken to the laboratory. The plasma-ascorbic acid was determined photometrically by the method of Mindlin and Butler (9).

The gonadotropic hormone used[2] is described as equine pregnancy serum standardized to contain 50 R.U. per cc. and was injected subcutaneously in one injection site in the region of the fore flank.

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[2] The authors wish to acknowledge the generous supply of equine gonadotropin (Gonadin) furnished by Donald H. Wonder, Director of the Endocrine Division, Cutter Laboratories, Berkeley, Calif., and the cooperation of Professor F. G. King and Mr. Gilman Stewart of the Animal Husbandry Department of Purdue University.

TABLE 1. EFFECT OF 1000 R.U. OF EQUINE GONADOTROPIN ON THE PLASMA-ASCORBIC ACID OF THE STEER

Steer	Blood Plasma-Ascorbic Acid									
	Hours before injection					Hours after injection				
	76	52	28	4	0	5	20	44	68	92
	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %
8	.583	.642	.393	.368	.430	.086	.347	.691	.548	.458
18	.395	.493	.527	.472	.454	.201	.118	.476	.417	.433
33	.583	.566	.575	.576	.557	.401	.301	.476	.543	.519
36	.455	.489	.471	.510	.464	.340	.362	.510	.509	.503
44	.448	.518	.451	.401	.489	.386	.345	.457	.588	.509
Mean	.493	.542	.483	.466	.479	.283	.294	.522	.521	.484

TABLE 2. EFFECT OF 2000 R.U. OF EQUINE GONADOTROPIN ON THE PLASMA-ASCORBIC ACID OF THE STEER

Steer	Blood Plasma-Ascorbic Acid									
	Hours before injection					Hours after injection				
	76	52	28	4	0	5	20	44	68	92
	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %
5	.523	.600	.565	.492	.590	.201	.192	.273	.433	.443
15	.551	.453	.441	.454	.489	.044	.212	.506	.489	.499
24	.531	.562	.565	.585	.459	.365	.285	.520	.518	.607
41	.387	.489	.460	.401	.540	.268	.222	.411	.632	.558
45	.417	.446	.451	.473	.505	.206	.275	.430	.489	.504
Mean	.462	.510	.496	.481	.516	.217	.237	.428	.512	.522

TABLE 3. TIME RELATIONSHIPS OF ASCORBIC ACID DISAPPEARANCE FOLLOWING INJECTION OF EQUINE GONADOTROPIN

Date	Hour	Blood Plasma-Ascorbic Acid				
		Steer				Mean
		1	3	26	30	
		mg. %	mg. %	mg. %	mg. %	mg. %
1/29	8 a.m.	.563	.578	.538	.553	.558
1/30	9 a.m.	.528	.617	.573	.577	.574
1/30	11 a.m.	.530	.632	.563	.607	.583
1/30	1 p.m.	.552	.578	—	.562	.564
1/30	3 p.m.	.501	.568	.568	.649	.571
1/30	5 p.m.	.526	.594	.506	.650	.569
1/31	8 a.m.[1]	.499	.547	.592	.592	.557
1/31	9 a.m.	.488	.564	.473	.387	.478
1/31	11 a.m.	.459	.563	.483	.362	.467
1/31	1 p.m.	.352	.278	.218	.122	.242
1/31	3 p.m.	.244	.277	.213	.283	.254
1/31	5 p.m.	.318	.213	.254	.300	.271
2/1	8 a.m.	.270	.329	.285	.239	.281
2/2	8 a.m.	.417	.548	.394	.304	.416
2/3	8 a.m.	.437	.488	.540	.489	.488

[1] Time of injection.

In the first experiment the plasma-ascorbic acid values of two groups of 5 steers each were determined at daily intervals prior to gonadotropin injection and at 5, 20, 44, 68 and 92 hours post injection. One group of animals received 1000 and the other 2000 R.U. of the hormone.

A group of 4 steers was used in a second experiment to determine more closely the relationship of time and ascorbic acid disappearance. Blood plasma-ascorbic acid determinations were made at 2-hour intervals on the day preceding gonadotropin injection and at 1, 3, 5, 7, 9, 24, 48 and 72 hours following injection.

RESULTS

Experiment 1. The average pre-injection level of ascorbic acid of the 5 steers receiving 1000 R.U. of gonatropin was 0.492 mg. per cent. The average ascorbic acid

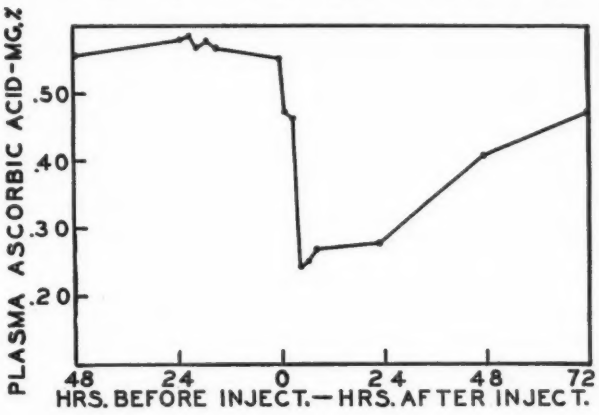

Fig. 1. Effect of equine gonadotropin upon venous blood plasma-ascorbic acid of the castrate bovine

value of the bloods drawn 5 hours post injection was 0.283 mg. per cent, a decrease of 42 per cent. In the group of 5 animals receiving 2000 R.U. of hormone the plasma-ascorbic acid dropped from an average pre-injection level of 0.497 to 0.217 mg. per cent within the same time interval, a decrease of 56 per cent. Pre-injection levels of plasma-ascorbic acid were reached 44 to 68 hours following gonadotropin administration. The data for individual animals are in tables 1 and 2.

Experiment 2. In order that the decline in plasma-ascorbic acid might be more closely followed, five blood samples were drawn from 4 steers during the first 9 hours following the injection of 2000 R.U. of gonadotropin. These data, which are presented in table 3 and figure 1, show that the maximum fall occurred during the first 5 post-injection hours and that return to pre-injection levels was definitely under way at the end of 9 hours. The average pre-injection level of ascorbic acid in this group was 0.567 mg. per cent and a decrease of 57 per cent occurred during the first 5 post-injection hours. This is comparable with the 56 per cent decrease which occurred in *experiment* 1 when 2000 R.U. of gonadotropin was administered.

DISCUSSION

The mechanisms involved in the reduction of venous blood plasma-ascorbic acid in the bovine following the administration of equine gonadotropin are not fully ex-

plained by this study but the findings add to the understanding of the ascorbic acid-gonadotropin relationship demonstrated by Erb and Andrews (7). The ovaries and testes undoubtedly have a rôle in this regulation in the intact animal, but the marked disappearance of ascorbic acid which occurred in the castrate bovine would indicate that gonadotropic hormone can act independently of the gonads in bringing about this reaction.

At least one significant difference was observed between the male and female bovine and the castrate; recovery of ascorbic acid to pre-injection levels was more rapid in the gonadectomized animals. Recovery required 8 to 10 days in the cow and longer in the bull unless extraneous vitamin C was injected, in contrast to recovery in 44 to 68 hours in the steer. This would seem to indicate that the effects of the injected gonadotropin on the gonads of the intact bovine were such that plasma-ascorbic acid levels were temporarily depressed.

Although pituitary assay studies were impossible in these experiments the depressing influence of injected gonadotropins upon the pituitary of the recipient should not be overlooked.

The report of Phillips et al (3, 4) that plasma-ascorbic acid levels are higher in estrus than in diestrus suggests that estrogens may influence the metabolism of vitamin C. The demonstration of Di Cio and Schteingart (8) that ascorbic acid augments the action of gonadotropin in the rat bears out our earlier suggestion that the frequent failure of gonadotropin therapy may be partially explained by the temporary decrease in plasma-ascorbic acid which follows the injection of the hormone.

SUMMARY

The injection of 1000 and 2000 R.U. of equine gonadotropin in the castrate bovine was followed by decreases in venous blood plasma-ascorbic acid of 42 and 56 per cent respectively within a period of 5 hours.

Recovery of plasma-ascorbic acid to approximate pre-injection levels required 44 to 68 hours.

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THE INFLUENCE OF CERTAIN HORMONES ON THE CARBOHYDRATE LEVELS OF THE CHICK

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A FEW SCATTERED OBSERVATIONS are recorded in the literature indicating that the endocrine regulation of carbohydrate metabolism in birds is quite different from that in mammals. Since a research program on the endocrine control of carbohydrate metabolism in mammals was being pursued in this laboratory, it seemed desirable to investigate some of these alleged differences in birds with a view to elucidating more fully the underlying regulatory mechanisms in either class of animals. An equally compelling reason for undertaking such an investigation at this time is the rapidly increasing usefulness of the chick (*Gallus domesticus*) in the biological laboratory. Quite apart from its economic importance, the chick is being widely employed for vitamin, hormone and virus research. Nevertheless, in spite of this widespread and growing use, only a relatively meager amount of information exists regarding avian carbohydrate metabolism. A brief review of this information is presented here.

The blood sugar. The blood sugar of a given species of animal is normally maintained between fairly narrow limits and at a level characteristic for that species. It has long been known that birds have a relatively high blood sugar level. In 1894 Weintraud (1) reported values for normal fasting ducks ranging from 117 to 198 mg. per cent. Since that time the higher values have been amply confirmed by other workers and this high level has been shown to be equally characteristic of other common bird species, namely, chickens, pigeons, geese and turkeys. The level of the blood sugar in birds is therefore about twice that of most common mammals whose blood sugar lies in the range 80 to 100 mg. per cent (somewhat lower in ruminants).

It is generally assumed that the sugar of the blood in birds is *d*-glucose. Evidence supporting this view was furnished as early as 1901 by Saito and Katsuyama (2) who prepared the osazone and demonstrated the fermentability of the blood sugar.

The blood sugar of birds decreases slightly with age (3, 4, 5). In chickens this is a change from about 200 mg. per cent in the young to 170 mg. per cent in the adult.

The arterial-venous difference in blood sugar content has been determined by Knapp (6). He found a difference of 17 mg. per cent in birds as compared with 8.5 mg. per cent in cattle.

Although Honeywell (7) was unable to detect any effects of fasting on the blood sugar of pigeons, this has not been generally confirmed. As will be shown later there is a slight but definite diminution of the blood sugar in chicks after a 24-hour fast.

Liver and muscle glycogen. As it is in mammals so in birds the chief deposit carbohydrate is the glycogen of the liver and skeletal muscles. Our knowledge of the normal stores of this material in the tissues of fasted and fed birds, of its formation and deposition and of its utilization is extremely limited. In fact, the few data relative to these points which do exist are open to serious question on technical grounds. It is now known that tissue for glycogen

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[1] The data forming the basis of this paper were taken from a dissertation submitted by W. R. C. Golden in partial fulfillment of the requirement for the degree of Doctor of Philosophy, Yale University, 1940.

analysis should be taken from an anesthetized but otherwise intact animal (8). Since this precaution was not observed in the various investigations recorded in the literature there is necessarily imposed a severe limitation on the interpretations and conclusions which may be drawn from data derived from such animals.

Silvette and Britton (9) have reported that red (leg) muscle contains only about one-third as much glycogen as white (pectoral) muscle. The actual values found were 0.24 and 0.89 per cent for red and white muscle, respectively. Noll and Becker (10) were unable to confirm the observations of Silvette and Britton. They found that the glycogen content of the breast muscle varied from 0.09 to 0.39 per cent and of the leg muscle from 0.04 to 0.31 per cent, thereby concluding that the difference is at most only very slight.

In fed chicks the level of liver glycogen is usually over 2 per cent and falls during a 24-hour fast to less than 0.5 per cent (11–13). Values for muscle glycogen both in fed and fasted chickens given by these authors are so variable as to be unreliable. Because of the manner in which the tissue was taken, the values are undoubtedly low. Information is also furnished by these authors on the glycogenic properties of various substances in chicks. It is interesting to point out that the first observation of the glycogenic property of dihydroxyacetone was made by Mostowski (14) in 1911.

Effect of pancreatectomy and insulin. It has long been known that the total removal of the pancreas in birds does not produce the typical diabetes seen in the dog and other mammals. When Minkowski in 1893 (15) published his now classical work on pancreatic diabetes in dogs he also described the effects of the removal of this gland from birds. Three pigeons and two ducks were completely depancreatized. The pigeons did not stand the operation well and died within 3 to 4 days. The ducks withstood the operation much better, showed an extreme avidity for food but subsequent poor digestion. In one of the ducks, the blood sugar 18 days after pancreatectomy was 136 mg. per cent. In spite of the administration of 15 gm. of glucose, no sugar could be demonstrated in the excreta. Although digestion was markedly impaired in the depancreatized ducks, they still possessed the ability to deposit in the liver large quantities of glycogen following ingestion of carbohydrate-rich food.

A year later Weintraud (1) described the results of pancreatectomy in a much larger number of birds. This investigator successfully depancreatized 19 ducks. In only 4 of the animals was a glycosuria produced. Of these 4 glycosuric birds, 3 died within a few days after the operation so that it was impossible to tell whether a permanent diabetes had been produced. The excretion of sugar was decreasing, however. The fourth survived 19 days and still exhibited a glycosuria at that time. The ducks showed no significant change in blood sugar level after pancreatectomy.

Kausch (16, 17) has described in some detail the operation and subsequent effects of removal of the pancreas in ducks and geese. The ducks withstood the operation better than the geese. Following the operation, usually within 24 hours, the blood sugar level was considerably elevated, attaining values as high as 700 mg. per cent and then gradually falling to slightly elevated levels. In many of the cases the elevation of the blood sugar was only moderate and in some hardly significant.

Glycosuria was seldom manifested, and then was only slight and transient. When sugar was administered there was only a slight excretion by the operated birds, indicating that they had utilized administered sugar. When very large doses were administered the blood sugar rose to a very high level, coma supervened and death occurred in this condition. If depancreatized ducks or geese were subsequently hepatectomized the blood sugar rapidly fell to undetectable quantities. In this respect the behavior was the same as in the depancreatized-hepatectomized dog. Kausch (16, 17) further ovserved that the ability of depancreatized ducks to increase liver glycogen after administration of glucose appeared to have been lost or greatly diminished. As had been reported by earlier investigators, the operated animals suffered chiefly from impairment of alimentary function, and soon grew emaciated.

Paton (18) has reported that in completely or partially depancreatized ducks no glycosuria appeared.

Fleming (19, 20) likewise observed no glycosuria in completely depancreatized ducks although these birds did manifest a rather elevated blood sugar. Similarly, Giaja (3) failed to

obtain a glycosuria in depancreatized adult male chickens although they also exhibited an increased blood sugar.

Although he observed an extreme hyperglycemia in pancreatectomized fowls, Kopannyi, et al (21) confirmed the temporary character of avian diabetes. Since it was not known whether the pancreas and other tissues of the chicken contained insulin, some of the tissues of normal chickens were assayed for their insulin content (22). The amount found in the pancreas was approximately the same as has been reported to occur in the pancreas of the calf. As a possible explanation for the transient character of avian diabetes, these workers suggested that insulin production is not specific for the pancreas with the result that sudden abolition of this source may cause a temporary diabetes until compensated for by increased production from the hypothetical alternate sources. This suggestion was later found to be untenable when Seitz and Ivy (23) found that they could extract no hypoglycemic material from the tissues of depancreatized ducks by a procedure which yielded such material, presumably insulin, when applied to the tissues of normal ducks. More recently Sprague and Ivy (24) have extended these observations on depancreatized ducks with essentially the same results as had been previously reported. These authors offer the suggestion that the avian pancreas when present functions in the regulation of carbohydrate metabolism in a manner similar to that of the mammalian pancreas, but its presence is not essential.

Shortly after insulin had become available for general experimental use, Riddle and coworkers (25, 26) reported that pigeons showed marked resistance to high doses of insulin and recovered without any manifestation of convulsions and insulin shock. Subsequent observations (27, 28, 29) in fowls have confirmed this phenomenon.

The influence of insulin on the distribution of glycogen in young chicks has been studied by Corkill (11). This author believed that a diminution of liver glycogen occurred in the insulin-treated chicks, although his data show no striking differences. When glucose was administered simultaneously with insulin, the usual accumulation of liver glycogen was prevented.

Influence of adrenal medulla and cortex. When epinephrine is administered to normal fed or fasted birds, or depancreatized birds a hyperglycemia and ensuing glysosuria is elicited 18, 20, 21, 29).

Because of the surgical difficulty, adrenalectomies have only rarely been performed in birds and these with doubtful success. Parkins (30) claimed to have removed completely the adrenal glands in fowls and reported a survival period of 38 to 146 hours for 18 fowls. Bilaterally adrenalectomized birds showed a lower blood sugar than unilaterally operated birds. After treatment with cortical extract the blood-sugar level rapidly returned to normal. The rapid fatality of birds following total adrenalectomy has been confirmed by Parkes and Selye (31) and Bülbring (32).

Herrick and Torstveit (33) achieved bilateral adrenalectomies in fowls by the use of cautery. Birds so operated were maintained on adrenal cortical extracts for as long as 82 days but eventually succumbed to the loss of the glands. Typical symptoms of cortical insufficiency did not appear but muscular weakness was evident before death.

Riddle, Dotti and Smith (34) have reported that adrenal cortical extracts injected into normal, and also hypophysectomized, thyroidectomized or partially adrenalectomized doves and pigeons increased blood sugar, on the average of 15 per cent at 7 hours after injection. There was also a slight increase in the basal metabolic rate of these birds.

Effect of hypophysectomy. In 1929 Mitchell (35) described the effects of hypophysectomy in Leghorn fowls and pointed out that removal of the gland was rapidly fatal to his birds. Confirmation of the extreme sensitivity of fowls to the loss of the pituitary was obtained by Hill and Parkes (36) and more extended observations were made by Hill, Corkill and Parkes (37). These workers found that 80 per cent of their totally hypophysectomized fowls died within 48 hours, but there was no indication that death was due to hypoglycemic shock. Treatment of the operated birds with adrenal cortical extract resulted in a significant prolongation of life. Administration of anterior pituitary extracts also resulted in a significant increase in survival time. Although there were insufficient data, the blood sugar appeared to be maintained at normal levels in the APE-treated birds. Limited observations on a few birds sug-

gested that hypophysectomized fowls do not show a greatly increased sensitivity to insulin.

In 1937 Riddle (38) reviewed the results of studies which he and his collaborators had made over a period of years on the carbohydrate metabolism of pigeons. The blood-sugar values of fasted hypophysectomized pigeons which Riddle has presented in this review appear to be only slightly lower than the values for fasting normal pigeons. Hypophysectomized mammals, by contrast, often show severe hypoglycemia when fasted. Contrary to the observations of Hill, Corkill and Parkes in the fowl, Riddle has stated that after hypophysectomy pigeons lose much, though not all, of their ability to withstand high doses of insulin. Anterior pituitary extracts containing prolactin increased the amount of histologically demonstrable glycogen in the livers of normal and hypophysectomized pigeons. Prolactin fractions contaminated with 'adrenotropic' factor greatly enhanced this effect. In a few hypophysectomized-depancreatized pigeons (so-called 'Houssay' birds) pure prolactin or very potent adrenotropic fractions produced a marked glycemia 3 days after the double operation. These doubly operated birds exhibited a somewhat elevated blood sugar, but not as high as those which were simply depancreatized. Riddle, in summation, makes the statement that " . . . All our observations indicate that carbohydrate regulatory mechanisms are essentially the same in birds and mammals."

In summary, the following facts relating to avian carbohydrate metabolism appear to be well established: *a*), the relatively high blood sugar in birds; *b*), the transient diabetes following pancreatectomy; *c*), the resistance to heavy dosage with insulin and *d*), the extreme sensitivity to hypophysectomy and adrenalectomy.

In the present experiments the carbohydrate levels of normal young chicks have been determined by established and reliable methods and the changes in these levels as effected by treatment with insulin, adrenal cortical extract, anterior pituitary extracts, and, in a limited number of cases, with epinephrine are reported.

EXPERIMENTAL PROCEDURES AND METHODS

Experimental animals. White Leghorn chicks were used throughout this investigation unless otherwise specified. They were obtained from a nearby commercial hatchery as 1-day-old chicks. Upon arrival at the laboratory they were placed in an electric battery brooder, in which they remained continuously until used for an experiment. The chicks were fed a commercial mash which, in addition to water, was always before them. The chicks attained a weight of 200 gm. at approximately 4 weeks of age. When it was desired to have fasted chicks for an experiment, the food was simply withdrawn but access to water was allowed. The beginning of the fast was taken as the time of removal of the food.

Chemical determinations. Glucose, derived either from blood filtrates or glycogen hydrolysates, was determined by the method of Shaffer-Hartman-Somogyi, employing 'reagent #2' as described by Peters and Van Slyke (39).

Blood for glucose determination was collected by direct bleeding from the neck vessels into small dishes containing sodium fluoride. The protein-free filtrate of the blood was obtained by Somogyi's 'zinc' methods (40).

Glycogen, in muscle or liver tissue, was determined by a modification of the procedure of Good, Kramer and Somogyi (41). The tissue was excised from chicks anesthetized with nembutal administered intraperitoneally in a dose of 50 mg. per kg. With the exception of one series of animals, in which both the gastrocnemius and pectoral muscles were taken for analysis, only a portion of the left pectoralis major muscle was taken for glycogen determinations. Following the removal of the muscle sample, the abdomen was opened and the cartilaginous caudal end of the sternum removed to make the liver easily accessible. One of the ventral lobes of the liver was gently grasped with a wide-tongued forceps, clamped off to prevent hemorrhage and

quickly cut away from the remainder of the liver. The chicks were always bled after the excision of muscle and liver tissue for glycogen determinations.

EXPERIMENTAL RESULTS

Carbohydrate levels of normal fed and fasted chicks. The values obtained for the carbohydrate levels of untreated fed and fasted chicks are presented in table 1. Although most of the subsequent data reported in this paper were obtained with chicks weighing about 300 gm., some chicks (*groups* 1 and 4, table 1) were considerably heavier and are therefore grouped separately. Actually, however, there appears to be no significant difference between the heavier birds and those in the 200-gm. range.

The data were examined for any significantly consistent difference associated with sex but none was found. Accordingly all groupings have been made irrespective of sex.

Attention is first called to the blood sugar values recorded in table 1. The high level of the blood glucose of birds is definitely confirmed by these values. Since they have been obtained on 'zinc' filtrates they probably represent values which are close to the true 'sugar' content of the blood. The decrease in blood sugar after a twenty-

TABLE 1. CARBOHYDRATE LEVELS OF NORMAL FED AND FASTED CHICKS

Group	Number of Birds	Liver Glycogen	Muscle Glycogen	Blood Glucose
		mg. %	*mg. %*	*mg. %*
1, Fed; weight over 300 gm.	8	2249±672	1168±64	192±6.3
2, Fed; weight under 300 gm.	30	2784±343	1093±34	191±3.4
3, Fasted 24-hr.; weight under 300 gm.	27	364±36	773±20	182±3.9[1]
4, Fasted 42–48 hr.; weight over 300 gm.	15	498±50	889±26	

[1] Mean for only 12 birds.

four-hour fast is rather slight, that is, less than 10 mg. per cent or about 5 per cent of the fed level.

The values for muscle glycogen are higher than those previously reported for this species. These relatively high values serve to substantiate the opinion that the results recorded in the early literature were vitiated by postmortal glycogenolysis. In the present work the analytical procedure for muscle glycogen was evaluated by determining the degree of reproducibility attainable with paired pectoral muscles. In 15 birds of *group* 3, table 1, in which this procedure was followed, the mean difference in the glycogen content of each pair of muscles, regardless of sign, was 43 mg. per cent or about 5 per cent of the total glycogen of the muscle. The respective means of the left muscle and right muscle independently differ by only 12 mg. per cent.

In view of the observation of Silvette and Britton (9) that pectoral (white) muscle contained more glycogen than leg (red) muscle, analyses were also made on the gastrocnemius muscle, which can be removed in entirety. The values obtained were very low, that is, some were under 200 mg. per cent. In contrast, the homologous muscle in fasted rats has a glycogen content of 500 to 600 mg. per cent. Since the pectoralis major is of larger size and easily accessible, a few preliminary analyses were made of the glycogen content of this muscle. The values obtained were about 3 times as high as those of the leg muscle. The actual mean values found in a group of 15 birds were 889 ±26 and 252 ±10 mg. per cent for the pectoralis major and gastrocnemius muscle, respectively.

The depletion of liver glycogen during a 24-hour fast from the fed level of approximately 3 per cent to a fasting level of less than 0.4 per cent is comparable to the changes which take place in rats under similar conditions.

Carbohydrate levels of fasted chicks after treatment with insulin. In the present investigation the birds were first fasted 24 to 30 hours. They were then given the insulin[2] in various amounts by subcutaneous injection. After the expiration of a fixed time interval the birds were anesthetized and muscle, liver and blood samples were obtained.

The data for the insulin-treated birds are presented in table 2. The liver glycogen of fasted chicks is definitely diminished by the injection of insulin and the magnitude

TABLE 2. EFFECTS OF INSULIN AND ADRENAL CORTICAL EXTRACT ON THE CARBOHYDRATE LEVELS OF CHICKS

Group, Treatment	Number of Birds	Liver Glycogen	Muscle Glycogen	Blood Glucose
		mg. %	*mg. %*	*mg. %*
1, Fasted controls, (table 1, *group* 3)	27	364±36	773±20	182±3.9[1]
2, 5 U/kg. of insulin; killed 2–2½ hr. later	8	192±23	844±41	49±6.5
3, 2 U/kg. of insulin; killed 3 hr. later	6	240±36	1024±21	69±9.4
4, 2 U/kg. of insulin; killed 2 hr. later	21	132±21	814±10	66±4.6
5, 1 U/kg. of insulin; killed 2 hr. later	15	154±32	763±24	87±5.4
6, Fasted controls; injected with saline or dilute epinephrine, 1:10[6]*	9	430±110	676±30	187±3.0
7, Fasted; cortical extract, 1 cc. per hour for 8–11 hours	13	1046±124	634±34	259±5.2
8, Fed controls; injected with saline	6	1520±377	957±77	193±4.4
9, Fed chicks; 1 cc. cortical extract per hour for 8–11 hours	6	1970±521	891±49	255±5.1
10, Cortical extract plus insulin; same as *group* 7, plus 5 U/kg. of insulin, 2 hr. before killing	7	426±54	805±54	88±7.5
11, Saline APE plus insulin; fasted 24 hr.; 1 cc. saline APE intramuscularly, 1–2 hr. before insulin; 2 U insulin per kg.; killed 2 hr. later	4	160	706	98±15
12, Saline APE plus insulin; 4–4.5 cc. of saline APE intramuscularly during 24-hr. fast. 2 U/kg. of insulin; killed 2 hr. later	15	244±24	906±49	57±3.2

[1] Mean for only 12 birds.
* Assay of the cortical extract for epinephrine: 1 part per million.

of the change is the same whether 5, 2 or 1 unit per kg. of body weight was given. Since a fraction of the material expressed as liver glycogen is actually 'non-sugar' reducing material, the low values recorded in table 2 are probably approaching levels of almost complete glycogen exhaustion.

With the exception of *group* 3 there is no significant alteration in the level of the muscle glycogen. In *group* 3, although there is an apparent elevation of the muscle glycogen, caution must be exercised in interpreting this increase as a positive effect of the insulin treatment. If this elevation of muscle glycogen is significant, it may have become apparent because of the allowance of an additional hour for the action of the insulin.

In considering the response of the blood sugar it must be admitted that the magnitude of the changes observed with these relatively small doses of insulin was not anticipated. Because of the well-demonstrated resistance of birds to the lethal effects

[2] The insulin was supplied by Eli Lilly & Co., Indianapolis Ind.

of large doses of insulin, the opinion has gained strength that small doses of insulin would not produce marked alterations in the blood sugar level. In these experiments as little as 1 U of insulin per kg caused a decrease of about 100 mg. per cent in the blood sugar within 2 hours. Even lower levels were attained with the higher doses. No significant difference is apparent between *groups* 3 and 4 in which the same dose, 2 U per kg., was allowed to act 3 and 2 hours, respectively. The minimum level of the blood sugar is probably reached within 2 hours of administration of the insulin but the limited data here make it impossible to ascertain at what point spontaneous recovery from the hypoglycemia occurred. In a few preliminary experiments on much larger birds, after rather heavy dosage with insulin (5 to 20 U), hourly blood samples were taken. In 5 of 6 birds so treated the lowest value for the blood sugar was attained at 2 hours and an increase had occurred in 4 of these 5 birds within the next hour.

Insulin tolerance. In view of the previous reports concerning the action of insulin in birds, it was considered of some interest to observe whether shock symptoms could be induced in the young chick by very large doses of insulin, since most of the previous experiments had been carried out on adult birds.

It was found that the individual responses of chicks treated with large doses (approximately 200 U per kg.) of insulin were quite unpredictable. In a few birds convulsions and death resulted from one large dose. What appears to be most significant, however, is that in several birds, although a single large dose did not cause convulsions, doses of the same or smaller size on successive days, produced shock and eventual death.

It is interesting to note in this connection that Corwin (42) recently observed in dogs a decreased resistance to insulin when given on successive days.

Effects of adrenal cortical extract on carbohydrate levels of fed and fasted chicks. In the present work the action of adrenal cortical hormone was studied first in fasted chicks. After the chicks had been subjected to a fast of 20 to 24 hours, they were given intraperitoneal injections of cortical extract[3] at hourly intervals for a period of 8 to 11 hours. The total amount of extract administered in this time was 8 to 11 cc. The birds were anesthetized 1 hour after the last injection and then muscle, liver and blood were obtained in the usual manner for glycogen and sugar determinations. At the time the birds were killed they had fasted a total of 30 to 34 hours.

Assay of the cortical extract used indicated the presence of epinephrine in an approximate concentration of one part per million. Therefore, fasted control birds were injected with 0.9 per cent saline alone, or containing epinephrine in this concentration. Otherwise they were treated in the same way as the cortin-injected chicks. The data of this experiment are given in table 2 (*groups* 6 and 7). It is apparent from this data that the adrenal cortical extract has effected a significant increase in the liver glycogen and has elevated the blood sugar to a supernormal level. There is no significant difference in the glycogen content of the muscles between the cortin-treated and control birds. Long, Katzin and Fry (43) have obtained essentially the same results in cortin-treated normal fasted rats. Although there was a slightly greater deposition of liver glycogen in their animals, the increase in blood sugar level was not as marked as in the chicks. In the rats the cortical extract raised the blood sugar to the level found in fed animals while in the chicks the blood sugar was raised above the fed level (cf. table 1, *group* 2). A level of 259 mg. per cent in the cortin-treated fasted chicks indicates an absolute increase of about 70 mg. per cent or a 40 per cent relative increase. The carbohydrate levels of the control birds which had been injected with the dilute

[3] This extract contains 10 per cent alcohol which was removed by vacuum distillation before use. The preparation was supplied by The Upjohn Co., Kalamazoo, Mich.

epinephrine or saline were essentially the same as those of normal, untreated, fasted chicks.

The administration of cortical extract to fed chicks was carried out in the same way as outlined above for the fasted chicks. While the birds were receiving the hourly injection of the extract they were left in their cages with access to food. Fed control chicks were injected with 0.9 per cent saline. The data of this experiment are also presented in table 2, *groups 8* and 9. While there is a higher level of liver glycogen in the cortin-treated chicks as compared with the saline controls it is not statistically significant. There is no significant difference in the muscle glycogen values of the cortin-treated and saline-control birds. The blood sugar in the fed birds treated with cortin was elevated about 30 per cent.

It is apparent from the above experiments that the cortical extract has effected changes in the carbohydrate levels of fasted chicks of an opposite character to those observed after insulin injection. Whereas the cortin brought about increases in liver glycogen and blood sugar, the insulin caused a diminution in liver glycogen and a rather sharp fall in blood sugar Since it has been reported (44) that cortical extract will ameliorate insulin convulsions in mice, a group of fasted chicks were given both cortical extract and insulin (table 2, *group 10*). It will be observed that the marked fall in liver glycogen that occurs with insulin alone has been checked by previous treatment with cortical extract and furthermore the blood glucose is not depressed to the same degree. Evidently the cortical hormones exhibit an anti-insulin effect in birds as well as mammals.

Effects of saline anterior pituitary extract on insulin hypoglycemia. Cope and Marks (45) and Young (46) have demonstrated that anterior pituitary extracts exert a contra-insulin effect in rabbits.

Several chicks were treated with a crude saline anterior pituitary[4] extract of known diabetogenic activity during a fasting period and were then given insulin in the usual manner. Under the conditions employed here there was no demonstrable effect of the pituitary extract. In the first group tested (table 2, *group 11*) the pituitary extract was given at the end of the fast, 2 hours before the insulin injection. The higher values of the blood sugar of these chicks over those of insulin controls (table 2, *group 4*) is probably without significance in view of the small number of birds in the group. Larger doses of the pituitary extract given at four intervals during the 24-hour fast likewise failed to produce any noticeable changes (table 2, *group 12*) when compared with the insulin-control birds. It would appear that in comparison to the mammal the 'glycotropic' action of crude pituitary extracts is not easily demonstrated in the chick.

Attempts to induce an elevation of the blood sugar in fed chicks with this saline extract were also without success.

Effect of epinephrine in fasted chicks. A limited amount of data has been obtained illustrating the glycogenolytic activity of epinephrine in chicks. The experimental procedure was similar to that described by Cori (47) for the study of muscle glycogenolysis in rats.

The procedure employed with the chicks was as follows. The birds were first fasted 24 to 30 hours. They were then anesthetized and a sample of left pectoral muscle was taken in the usual manner for glycogen determination. Immediately after the excision of the tissue the epinephrine was administered by subcutaneous injection and allowed to act for a fixed time interval. At the end of this period a sample of right

[4] The extract was prepared by grinding fresh ox anterior lobes with 0.9 per cent saline. The mixture was centrifuged and the supernatant fluid removed and kept frozen until used. Its 'diabetogenic' activity was demonstrated in the partially depancreatized rat.

pectoral muscle was taken from the bird which was still under anesthesia. In some of the birds it was also possible to obtain a blood sample from the wing vein before administering the epinephrine and a terminal blood sample from the jugular vein after the excision of the second muscle sample. In a few birds, a portion of liver was also excised after the second muscle. In such cases if blood was also being taken the liver sample was taken first.

The first group of chicks were given a dose of 0.2 to 0.3 mg. of epinephrine per kg. and it was allowed to act for 30 to 35 minutes (table 3, *group 2*). This was the dose which Cori had employed in the rat. No significant or regular changes in the muscle glycogen were observed to follow this dosage and time interval. The blood sugar did not appear to be affected at all under these conditions. In the next series of birds (table 3, *group 3*) the dosage was increased to 0.5 mg. per kg. and the time of action was extended to 1 hour. The difference between the mean value for muscle glycogen

TABLE 3. EFFECT OF EPINEPHRINE ON CARBOHYDRATE LEVELS OF FASTED CHICKS

Group, Treatment	Number of Birds	Liver Glycogen	Muscle Glycogen		Blood Glucose	
			Before	After	Before	After
		mg. %	mg. %	mg. %	mg. %	mg. %
1, Controls; 1 cc. 0.9% saline, 1 hr.	4	449±82	910±56	925±64	162[1]	189±14.6
2, Epinephrine; 0.2–0.2 mg./kg., 30–40 min.	5		922±56	869±33		188±10.8
3, Epinephrine; 0.5 mg./kg. 1 hr.	15	363±81	849±22	687±31	155±8.7[2]	224±5.8

[1] One Bird.
[2] Mean of 3 birds.

before epinephrine and the mean value after epinephrine was slightly over 160 mg. per cent. In 9 of the 15 cases in this group the decrease in muscle glycogen was approximately 200 or more mg. per cent. There was a significant but not great increase in the blood sugar value. Initial blood sugar values were obtained from only 3 birds and since they are a little lower than usual, the apparent increase of 70 mg. per cent is probably too high; an increase of 40 to 50 mg. per cent is probably more nearly right. In the few birds in which liver glycogen determinations were carried out, there did not appear to be any noticeable effect of the epinephrine.

DISCUSSION

In reviewing the work of previous investigators who have published values for muscle glycogen in birds, it has been pointed out that values recorded were probably too low because the methods employed had invalidated the results. Sufficient justification for this opinion is now furnished by the values for muscle glycogen which were obtained in the present work by removing the tissue from anesthetized but otherwise intact birds. These values are not only considerably higher than the previously published ones, but are also of sufficient uniformity and reproducibility that the difference between the levels in fed and fasted birds can be easily demonstrated. It is worth noting in this respect that Schmitt-Kramer (48) who studied the postmortal formation of lactic acid in different types of muscles published maximum lactic acid values for fowl's muscles which exceeded the glycogen values of subsequent investigators. Apparently this work had been overlooked, for had sufficient cognizance of it been taken, investigators might have been led to suspect their low glycogen values. Schmitt-Kramer also furnished additional indirect evidence, from the lactic acid

values, that pectoral muscle contains normally considerably greater stores of glycogen than the leg muscles.

Corkill's observations that insulin causes a decrease in the already low level of the liver glycogen of the fasted chick have been confirmed. It is worth pointing out that he administered the insulin in doses of 80 U each to birds that weighed probably 1 to 1.5 kg. while in the present work as little as 1 or 2 U of insulin per kg. produced the observed changes. It is of further interest that the low blood sugar values (52 to 84 mg. per cent) which Corkill obtained in these heavily insulinized birds were not significantly different from the values obtained here with 2 U per kg. The question of whether insulin increases the muscle glycogen in chicks cannot be adequately answered from the present data. Although Corkill claimed that it did cause a small increase in muscle glycogen, his data do not seem to justify that conclusion. In the present experiments the small increases in muscle glycogen, particularly noted in one group of birds in which the muscles were taken for analysis 3 hours after administration of the hormone, may be suggestive, though not conclusive, of the positive effect of insulin in this direction.

It had been demonstrated that adrenal cortical extract not only increases the liver glycogen of fasting chicks, but also raises the blood sugar to supernormal levels without, however, affecting the muscle glycogen. In considering these increases the question naturally arises as to the source of this extra carbohydrate. Evidence has accumulated from several sources that in mammals the adrenal cortex stimulates the formation of new carbohydrate from protein. On *a priori* grounds it may be assumed that the adrenal cortical extract has a similar action in the chick. Although there is no direct evidence from the present experiments on chicks, it can be shown by calculation from the data in this paper that this assumption is not an unreasonable one. In a 200-gm.-fasted chick, treated with cortical extract, the liver glycogen increases approximately 613 mg. per cent and the blood sugar 72 mg. per cent (table 2). The liver weight of such a chick is approximately 6 gm. and therefore there is an increase of 37 mg. of liver glycogen. A 200-gm. chick has about 140 gm. of body water[5] and if it is assumed that the sugar is evenly distributed in it[6] there is an additional amount of 100 mg. of extra carbohydrate in the body fluids. That gives a total of 137 mg. of extra carbohydrate to account for in a fasting chick treated with cortical extract. If it were not produced from some non-carbohydrate source then it would have to come almost entirely from the muscle glycogen. In a 200-gm. chick there is approximately 50 gm. of muscle. If 137 mg. of carbohydrate is to be furnished by 50 gm. of muscle there must be an average decrease of 274 mg. per cent in the glycogen content of the muscle. Actually the values for the muscle glycogen are about the same in the cortin-treated birds as in the saline-treated controls. Accordingly it would seem safe to assume that the extra carbohydrate was produced from a non-carbohydrate source, presumably protein.

SUMMARY

1. The carbohydrate levels of normal fed and fasted chicks have been determined. The blood-sugar level in the fed chick is about 200 mg. per cent and decreases about 10 mg. per cent on fasting. This value confirms the earlier findings that in birds the

[5] Direct determination of the total body water content in 2 chicks gave 73 per cent in each. Determination of the percentage of body weight accounted for by skeletal muscle gave approximately 25 per cent. On the basis of this data a 200-gm. chick would have approximately 140 gm. of total body water, and 50 gm. of skeletal muscle.

[6] This assumption may involve some error in view of the absence of any accurate information on the sugar content of the tissues. In rats it is generally assumed that the glucose is evenly distributed in half the body weight. If we make such an assumption for the chick there will be an apparent smaller increase in sugar of the body fluids.

normal blood sugar level is twice that of mammals. The muscle (pectoral) glycogen in the fed chick is over 1000 mg. per cent and declines to 750 to 800 mg. per cent during a 24-hour fast. The pectoral muscle has been shown to contain 3 to 4 times more glycogen than the gastrocnemius muscle.

The liver glycogen of the fed birds, though quite variable, averages about 3.0 per cent and declines to levels below 0.4 per cent during a 24-hour fast.

2. In the fasted chick small doses of insulin (1 to 2 U per kg.) cause a definite hypoglycemia and a diminution of the already low level of liver glycogen.

Insulin convulsions and eventual death of the chick could be produced by rather high dosage of the hormone if administered on successive days. The response of the chicks however was irregular and unpredictable.

3. Adrenal cortical extract administered to fasted chicks produces a significant deposition of liver glycogen and raises the blood sugar to supernormal levels. In fed chicks the same elevation of the blood sugar occurs but no alteration in the liver glycogen is observed.

It is suggested that protein is the source of the new carbohydrate formed after administration of adrenal cortical extract to fasted chicks.

4. No effect of saline anterior pituitary extract on the blood sugar of fed chicks could be demonstrated, nor could any 'contra-insulin' action be shown in fasted chicks.

5. The hyperglycemic and glycogenolytic properties of epinephrine have been shown in the chick but no evidence was adduced to indicate that the chick is any more sensitive than mammals to the action of this hormone.

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INFLUENCE OF HYPOPHYSECTOMY ON THE ABILITY OF THE RAT TO REMOVE FRUCTOSE FROM ITS BLOOD[1]

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GOETSCH, CUSHING AND JACOBSON (1) found that the amount of fructose needed to cause fructosuria when given by mouth was increased in man in clinical states diagnosed as primary hypopituitarism. Rowe et al (2), however, found that this type of fructose tolerance was not characteristically altered in their series of cases of pituitary hypofunction. The study reported here was undertaken to explore further the possible relationship of the pituitary gland to fructose metabolism.

EXPERIMENTAL PROCEDURE

Adult, male, albino rats hypophysectomized by a parapharyngeal route were matched with controls given a sham operation which was identical except that the gland was not removed. The sellae turcicae together with a layer of overlying brain were sectioned serially to prove the absence of all gland normally lying below the diaphragma sellae. The results from any hypophysectomized animal in which a fragment remained were discarded. All of the animals were maintained by feeding by stomach tube, beginning 3 days before the operation in order to eliminate as a factor the cachexia regularly occurring after hypophysectomy. Eighteen cc. per day of the diet presented in table 1, given in two feedings, were found to be sufficient to cause a moderate gain in weight in both the hypophysectomized animals and their controls as is shown in figure 1. No more food was given after the morning feeding on the

TABLE 1. DIET[1]

Powdered ovalbumin	291.7 gm.	Glucose monohydrate (Cerelose)	110.0 gm.
Lactalbumin (Labco #16)	41.7 gm.	Roughage (Cellu-Flour)	167.0 gm.
Melted butter	377.0 gm.	Vitamin B complex (Squibb)	10 capsules
Corn oil (Mazola)	30.0 gm.	Vitamin A & D (Percomorph Oil)	67 drops
Cornstarch	207.0 gm.	Osborne-Mendle salt mixture (Harris)	66.6 gm.
Dextrin N.F. V	100.0 gm.	Water sufficient to make a total volume of	2000.0 cc.

[1] For details see a previous report (3). Since this was published it has been found that the 'Record' type of syringe is much superior to the all-glass type for this kind of diet. The rigid type of stomach tube (4) has been tested and found to be impractical in our hands for long-term feeding regimes. The #8 french, rubber catheter has been found to make a satisfactory stomach-tube, but the use of a gag as mentioned in our report and lately by others (5) has proved to be so unsatisfactory that it has been abandoned in favor of removing the points of both upper and lower incisors with a small pliers. The movements of the tongue and mandible can then be left unrestricted during the passage of the tube. This both facilitates the passage of the tube and allows the animal to prevent its entry into the larynx. In addition the procedure is simplified to such an extent that one person can easily carry it out with relatively little previous experience. Inexperienced assistants regularly performed this task quite satisfactorily after brief training.

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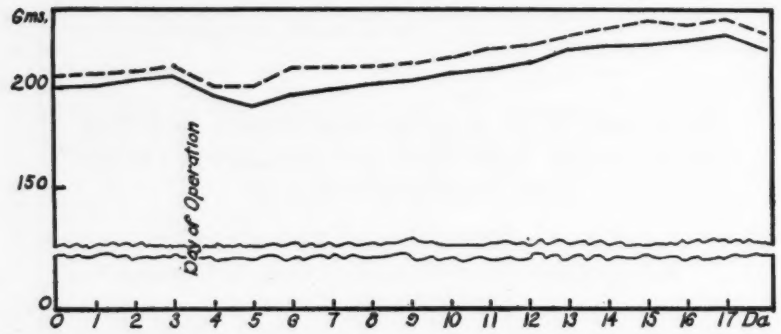

Fig. 1. Maintenance of body weight in the hypophysectomized rat by feeding by stomach tube. *Solid line* represents average weight of hypophysectomized animals; *broken line*, the average weight of control animals. The animals were weighed daily before the morning feeding. The loss of weight on the day of operation is at least partly explained by the fact that no food was given on that day. The loss of weight on the 18th day was due to the omission of the evening feeding on the 17th day.

14th day after hypophysectomy. At noon the next day, each animal was given via the saphenous vein 0.25 cc. of a 50 gm. per cent solution of Pfanstiehl's 'C. P. Special' fructose per 100 gm. of body weight. Samples of 0.05 cc. of blood were taken from the tips of the animals' tails before and at approximately 10, 20, 30, 60, 120, 180 and 240 minutes after the injection of the sugar. One-tenth mg. of phenol red per 100 gm. of body weight was given intravenously shortly before the last sample of blood was taken. The urine of each animal was collected separately for 8 hours after the injection of fructose.

The blood samples were analyzed for fructose by the skatole color reaction (6) and for total sugar by a modification of the method of Jeghers and Myers (7) which had been shown to give the same values for both glucose and fructose. The difference between the fructose and total sugar values for a sample was taken as its glucose content.

The urine samples were rinsed into 100 cc. volumetric flasks containing 10 cc. of 10 per cent zinc sulfate. A saturated solution of barium hydroxide was then added until the phenol red, which had been excreted without exception by all of the animals, turned pink. The samples were next diluted to volume with water and a portion of each centrifuged. The supernatant fluid was decanted and acidified with a trace of concentrated sulfuric acid. Any barium sulfate formed was removed by recentrifugation. The fructose in the clear fluid was then estimated by the method mentioned. Known amounts of fructose added to urine were determined with satisfactory accuracy by this procedure.

RESULTS

The results of the blood analyses are shown in figures 2 and 3. Similar experiments on smaller groups from 5 to 32 days after hypophysectomy showed essentially the same results. The blood fructose level was found to be slightly higher in the hypophysectomized animals during the first hour. This proved to be a statistically significant difference and raised the question, 'Is the hypophysectomized animal able to remove fructose from its blood stream as rapidly as the control?' Inspection of the fructose curves shows that the rate at which the fructose decreases is a function of the fructose concentration. This makes it necessary to compare the rates of decrease for the two groups at the same concentration, which, because of the nature of the data, is difficult. Therefore, the fructose curves for the first 60 minutes after the injection

were replotted on semilogarithmic graph paper (fig. 4). In this form the curves in both groups approximate straight lines showing that the equation $C = C_0 10^{-kt}$ holds

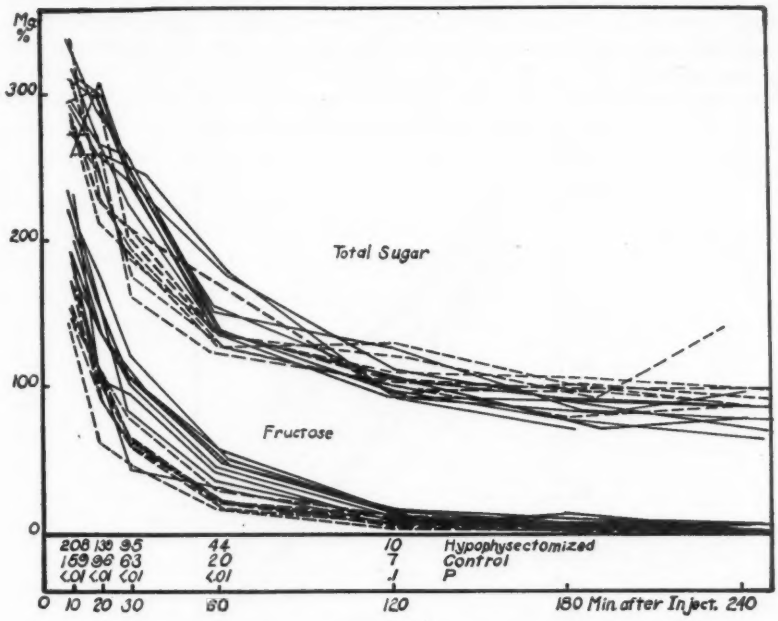

Fig. 2. CONCENTRATION OF FRUCTOSE AND TOTAL SUGAR IN THE BLOOD AFTER THE INTRAVENOUS ADMINISTRATION OF 125 MG. OF FRUCTOSE PER 100 GM. OF BODY WEIGHT. *Solid lines* indicate hypophysectomized animals; *broken lines*, control animals. The mean fructose levels for the hypophysectomized and control animals for the first 120 minutes are shown at the bottom of the figure. The values of *P* given are those obtained by the *t* test as calculated to determine the significance of the difference between these means. A value of less than .05 is generally assumed to indicate that the difference between the means is significant (8).

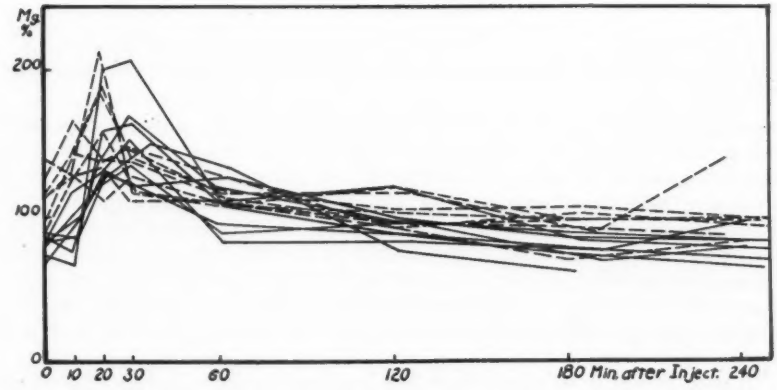

Fig. 3. CONCENTRATION OF GLUCOSE IN THE BLOOD AFTER THE INTRAVENOUS ADMINISTRATION OF 125 MG. OF FRUCTOSE FOR 100 GM. OF BODY WEIGHT. *Solid lines* indicate hypophysectomized animals; *broken lines*, control animals.

approximately for both. C equals the concentration of fructose at any point on the curve, C_0 the original concentration of fructose, t, the time between the taking of C_0 and C, and k, a measure of the slope of the curve as plotted on semilogarithmic graph paper. Since the curves for the two groups as shown on figure 4 seemed to diverge somewhat, the value of k was calculated for each curve, the concentration at 10 minutes being taken as C_0 and at 60 minutes as C. The concentrations for these times were used because the result obtained from them would be least affected by

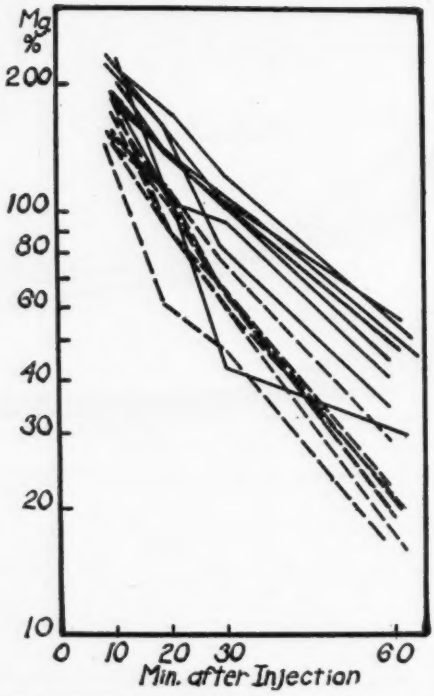

Fig. 4. CONCENTRATION OF FRUCTOSE IN THE BLOOD AFTER THE INTRAVENOUS ADMINISTRATION OF 125 MG. OF THAT SUGAR PER 100 GM. OF BODY WEIGHT. *Solid lines* indicate hypophysectomized animals; *broken lines*, control animals. Data from figure 2 for the first 60 minutes as replotted on semilogarithmic graph paper.

experimental error. The value of k thus obtained for each curve can be thought of as a measure of the slope of a straight line drawn through its first and last points as plotted in figure 4. The mean value of k for the hypophysectomized group was .0136 as compared with .0179 for the controls. The value of P (see legend for figure 2) was found to be less than .01 showing that the divergence was significant and indicating that hypophysectomy did cause some reduction in the rate at which the rat removes fructose from its blood. A sober consideration of the data, however, leads to the conclusion that this is only a small reduction and may easily be a secondary result of other changes, for example a decrease in the rate of circulation of blood through the liver.

It is of interest to note that others have obtained similar curves (9) when they have plotted the bloodsugar values obtained after the intravenous administration of glucose on semilogarithmic graph paper. It seems probable, therefore, that both sugars, when they are present in the blood in excess, are removed by processes which

follow similar chemical and physical laws. That this removal is chiefly a function of the liver and kidneys is indicated by the fact that in the eviscerated, nephrectomized rat the removal of both of these sugars is not only much slower but at a rate that is independent of their concentration (10).

The increase in blood glucose found after the intravenous injection of fructose agrees with the finding for the fasting dog (11) and disagrees with that for the fasting rabbit (12). Hypophysectomy apparently does not markedly alter this effect. The significantly lower level of the blood glucose in the hypophysectomized animals at the beginning of the experiment (average 80 mg.% as compared with 114 mg.% for the controls; $P < .01$) agrees with the finding for the fasting hypophysectomized rat which has been fed ad libitum (13).

The results of the urine analyses shown in table 2 apparently indicate that hypophysectomy also causes some reduction in the ability of the rat's kidney to excrete

TABLE 2. MILLIGRAMS OF FRUCTOSE EXCRETED IN URINE

Hypophysectomized Rats	Control Rats
41	52
38	48
32	48
29	46
28	44
22	42
19	24
14	4[1]
Mean 28	43
P[2] .01	

[1] Omitted from calculation of average and P because of abnormal deviation.
[2] See legend of figure 2.

fructose, although this also seems to be a small effect. Stasis of urine containing fructose in the urinary passages was eliminated as a factor by the excretion of phenol red into all of the samples, for this dye was injected after the blood fructose level was already low and the further excretion of fructose therefore unlikely.

SUMMARY

Evidence has been presented which indicates that hypophysectomy does reduce the rate at which the rat removes intravenously administered fructose from its blood stream, but this reduction is so small that it may be stated that the lack of pronounced effect of hypophysectomy on the rate of removal of fructose from the blood is the more impressive fact.

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CHANGES IN BLOOD CHEMISTRY ASSOCIATED WITH CIRCULATORY FAILURE IN THE ADRENALECTOMIZED DOG

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THE HEALTHY ADRENALECTOMIZED dog will develop a fatal circulatory collapse within 5 to 12 hours after being subjected to any of a wide variety of types of stress. This peripheral vascular failure can be prevented by the administration of liberal amounts of cortical extract. When cortical extract was replaced by crystalline adrenal steroids, it became apparent that the action of these steroids was qualitatively dissimilar. Desoxycorticosterone acetate proved effective in preventing the circulatory failure following hemorrhage, the vascular dehydration accompanying intraperitoneal glucose injections and muscle trauma. It was ineffective after intestinal stripping and bilateral adrenalectomy at a single stage operation (1, 2). Compound *E* of the Kendall series (17-hydroxy-11-dehydrocorticosterone) was an excellent prophylactic against the circulatory failure following the latter two procedures (3), and also after muscle trauma and hemorrhage. The experiments do indicate, however, that desoxycorticosterone acetate was more effective after hemorrhage than was compound *E*, and had a different qualitative action.

This discrepancy between the actions of desoxycorticosterone acetate and compound *E* could not be given a rational explanation until it was discovered that priming pre-treatment with desoxycorticosterone acetate would not protect against circulatory failure following muscle trauma unless the animal had been previously treated with natural extract (3). The small quantities of extract, quite insufficient in themselves to protect against circulatory failure, enabled desoxycorticosterone acetate to exert its protective influence. The explanation immediately suggested is that desoxycorticosterone acetate, which apparently lacks influence on carbohydrate metabolism, is dependent upon the presence of small amounts of carbohydrate-active steroids. The underlying derangement in circulatory failure could therefore be a failure of the vasculature (along with other tissues) to properly metabolize carbohydrate, which in turn would affect its functional capacity.

Before the correlation between the carbohydrate activity of the various steroids and their ability to protect against circulatory failure could be accepted as more than coincidental, it seemed essential to ascertain whether other known physiological actions of the various adrenal hormones, such as the influence on renal function and electrolyte and fluid distribution in the body, could be influencing the results in any way. In an attempt to clarify this point, the results of blood analyses have been collected on a series of dogs subjected to stress procedures and treated with various adrenal hormones.

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[1] Upjohn Research Fellow.

METHODS

The data to be presented were collected from a series of 59 experiments on adrenalectomized dogs maintained in normal physical condition by adequate daily doses of cortical extract or desoxycorticosterone acetate. Four stress procedures were employed: *a*), visceral trauma, as produced by a 25-minute gentle stripping of the small intestine; *b*), surgical trauma, as embodied in the removal of both adrenal glands at a single operation, no undue care being taken to avoid injury to adjacent nervous tissue; *c*), trauma to muscle masses, by 100 blows to the thigh muscles of one leg with a wooden mallet; and *d*), simple hemorrhage by femoral puncture, continued until the arterial pressure had been reduced to 40 to 50 mm. Hg. The details of these techniques, as well as the methods used for chemical analyses, have been reported previously (1–5). The adrenal hormones tested were cortical extract, desoxycorticosterone acetate, corticosterone, and compound *E*. Desoxycorticosterone acetate was given intramuscularly in oil, in divided doses, 16, 12 and 2 hours before the experiment. Corticosterone and compound *E* were given intravenously in 50 per cent alcohol, 5 mg. at 2 hours before, and 2, 5, and 8 hours after the start of the experiment (3).[2,3]

RESULTS

The essential data have been grouped in tables 1 to 4, all figures given being the averages of a group of animals receiving similar treatment. Unless otherwise mentioned, the changes indicated were consistent, at least in direction, for all animals within a series.

Blood concentration. One of the most consistent features of adrenal insufficiency is a rise in hematocrit and hemoglobin levels, indicating a progressive vascular dehydration (6–8). This dehydration can be attributed to a fluid loss through the kidneys, to a shift of water into intracellular regions of the body, and, perhaps, to an unequal distribution between phases of the extracellular compartment. Cortical extract will correct all factors and restore the reduced blood volume to normal. Desoxycorticosterone acetate will not only restore the volume, but will increase it above normal in both adrenalectomized and intact animals (4). This action is probably referable to an altered kidney function, leading to a marked positive water balance. The action of corticosterone and compound *E* on the blood volume are not known, but they are presumably far less effective than desoxycorticosterone acetate.

Only two of the four stress procedures employed induced a hemoconcentration, muscle trauma and visceral trauma (tables 1–4). That following visceral trauma tended to be more severe and was not corrected by any therapy used. The concentration following muscle trauma was less constant in degree, and there is some evidence that desoxycorticosterone acetate was effective in delaying, and also in relieving the hemoconcentration in the later hours. Compound *E* had no effect in this regard. Bilateral adrenalectomy at a single operation was not followed by significant hemoglobin changes. Hence, hemoconcentration was involved only in those stress procedures which were accompanied by an appreciable local loss of fluid, and was an index to the severity of the tissue abuse leading to a sequestration of blood and loss of plasma. No clear correlation between the degree of concentration and the onset of circulatory collapse has been observed.

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[2] We are greatly indebted to Dr. E. C. Kendall Division of Biochemistry, Mayo Foundation for generous supplies of Compound *E*.

[3] The corticosterone was obtained from Dr. J. J. Pfiffner, Parke, Davis and Co., Detroit, Mich., and Dr. Oscar Wintersteiner, Squibb Institute for Medical Research, New Brunswick, N. J. Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products Inc., Summit, N. J., furnished the desoxycorticosterone acetate (Percorten).

After hemorrhage, as to be expected, there was an actual blood dilution as partial compensation for the experimentally reduced blood volume. In animals treated with desoxycorticosterone acetate, this hemodilution started at the time of the first removal of blood, and continued so rapidly that a full half of the total dilution was present by the 3rd hour (table 4). Compound *E*-treated animals responded just as did untreated dogs, with no dilution until after the 3rd hour, when it progressed slowly

TABLE 1. EFFECT OF ADRENAL HORMONES ON RESPONSE OF THE ADRENALECTOMIZED DOG SUBJECTED TO VISCERAL TRAUMA

Hours	Blood Pressure, mm. Hg	Pulse, /min.	Hemoglobin, gm. %	Blood Urea Nitrogen, mg. %	Blood Sugar, mg. %	Serum Sodium, m. eq./l.	Serum Chloride, m. eq./l.	Serum Potassium, m. eq./l.
				Untreated, 5 Dogs				
0	105	86	11.8	23.4	88	142.0	111.5	5.9
5	76	181						
8	45	161	13.9	27.9	72	141.9	113.3	5.6
11	All dead or revived with cortical extract							
				Cortical extract, 40 cc., 1 Dog				
0	112	120	10.3	18.0	81	142.5	110.6	5.2
5	86	196			84			
8	98	184	13.8	18.2	100	143.1	112.4	5.3
11	96	168	13.4		89			
24	110	130	13.1	16.4	88	143.0	111.6	5.5
				Desoxycorticosterone acetate, 15 mg.; 3 Dogs				
0	112	60	7.1	20.8	89	144.6	115.0	6.0
5	64	200			75			
8	42	168	11.5	25.0	62	143.2	113.4	5.7
11	All dead							
				Corticosterone, 20 mg.; 3 Dogs				
0	107	79	11.0	22.2	80	141.6	109.9	5.1
5	111	136			86			
8	98	160	14.9	49.0	86	141.5	109.3	5.0
11	88	184	16.9	63.0	73	141.5	109.3	5.0
24	93	192	15.3	62.5	70	135.5	104.2	5.1
				17-hydroxy-11-dehydrocorticosterone, 20 mg.; 4 Dogs				
0	107	106	12.4	18.0	84	144.7	110.3	5.5
5	99	172	14.6		75			
8	103	156	14.6		89			
11	101	157	14.6	18.0	97	140.6	109.4	4.6
24	106	146	14.4	21.3	92	139.6	105.1	5.1

but steadily throughout the experimental period. This difference in dilution rate, as well as the difference in initial pressure response to the bleeding (2), could perhaps best be explained by assuming that desoxycorticosterone acetate-treated animals, unlike compound *E*-treated and untreated ones, reacted to the hemorrhage by an intense vasoconstriction.

The ability of the desoxycorticosterone acetate-treated animal to maintain this initial vasoconstriction, and to resist the development of circulatory failure, depends

in large part on the therapy used for maintenance prior to the experiment. As shown in table 4, 3 of 4 dogs given large amounts of desoxycorticosterone acetate after this steroid had been used for maintenance for at least 10 days (0.5 mg. per day), withstood

TABLE 2. EFFECT OF ADRENAL HORMONES ON THE RESPONSE OF THE ADRENALECTOMIZED DOG SUBJECTED TO SINGLE STAGE BILATERAL ADRENALECTOMY

Hours	Blood Pressure, mm. Hg	Pulse, /min.	Hemoglobin gm. %	Blood Urea Nitrogen mg. %	Blood Sugar, mg. %	Serum Sodium m. eq./l.	Serum Chloride, m. eq./l.	Serum Potassium, m. eq./l.
Untreated, 4 Dogs								
0	110	102	14.2	14.0	87	145.8	110.9	5.7
5	94	126	14.0	14.0	79			
8	69	158	14.2	13.4	70			
11	50	144	14.3	14.4	62	138.3	107.3	5.8
24	All dead or revived with cortical extract							
Untreated. Novocaine infiltration, 3 Dogs								
0	110	84	15.5	14.2	84	143.0	110.9	6.5
5	115	104			78			
8	100	162			72			
11[1]	100	160	15.6	15.5	69	143.5	111.4	6.0
Cortical extract, 30 cc. 2 Dogs								
0	113	120	14.1	12.1	81	148.1	115.8	4.7
5	121	130	14.2	14.2	86			
8	77	128	13.8	17.1	90			
11	68	156	13.9	20.6	90			
24	91	124	14.0	23.1	80	142.1	111.0	4.9
Desoxycorticosterone acetate, 20 mg., 4 Dogs								
0	110	93	14.0	16.6	86	144.3	111.1	5.5
5	96	150	14.0	17.0	80			
8	47	147	14.8	18.9	57	143.2	109.2	5.7
11	All dead or revived with cortical extract							
Corticosterone, 20 mg., 1 Dog								
0	105	120	13.8	9.0	87	144.2	108.1	5.3
5	83	184	14.0		90			
8	87	240	13.8		107			
11	80	240	13.9	12.7	96			
24	98	160	13.7	11.0	94	136.1	103.6	6.2
17-hydroxy-11-dehydrocorticosterone 10–20 mg., 4 Dogs								
0	116	101	13.9	12.1	86	145.2	112.7	4.8
5	123	157	13.2		79			
8	116	149	13.5		89			
11	114	131	13.6	10.5	96	139.2	112.7	4.8
24	114	120	14.0	13.2	83	136.7	105.8	5.8

[1] Given maintenance levels of desoxycorticosterone acetate at the 12th hour.

the loss of less blood before the pressure was reduced to shock level, showed but little pressure rise from this level and succumbed within a few hours. It would seem that the initial period of vasoconstriction had, in fact, precipitated the circulatory col-

lapse. When, however, desoxycorticosterone acetate was given animals which had been previously maintained on whole cortical extract (3–5 cc. per day), full protection against circulatory failure was afforded, and the pressure was usually returned to normal within 24 hours. The amounts of cortical extract given cannot, in themselves, give observable protection against circulatory collapse.

Blood urea nitrogen. A second feature of adrenal insufficiency is a rise in blood urea nitrogen, a change which is extremely sensitive to a decrease in hormone levels

TABLE 3. EFFECT OF ADRENAL HORMONES ON THE RESPONSE OF THE ADRENALECTOMIZED DOG SUBJECTED TO MUSCLE TRAUMA

Hours	Blood Pressure, mm. Hg	Pulse, /min.	Hemoglobin, gm. %	Blood Urea Nitrogen, mg. %	Blood Sugar, mg. %	Serum Sodium, m. eq./l.	Serum Chloride, m. eq./l.	Serum Potassium, m. eq./l.
Untreated. 5 Dogs								
0	104	83	12.0	14.8	83	141.2	111.9	5.5
5	72	157						
8	52	169	14.2	19.2	69	141.8	113.7	7.1
11	All dead or revived with cortical extract							
Desoxycorticosterone acetate, 10–20 mg. 4 Dogs on maintenance desoxycorticosterone								
0	116	91	8.7	24.0	85	146.2	113.5	4.8
5	45	174	8.8	29.1	64	144.8	113.6	5.1
8	3 of 4 dead, other survived 24 hours							
Desoxycorticosterone acetate, 10–20 mg. 3 Dogs on maintenance cortical extract								
0	110	90	11.2	26.5	86	144.7	112.0	5.2
5	98	159	11.2	28.4	93			
8	86	165	11.9	32.1	92			
11	88	159	11.3	34.0	86	143.5	107.7	5.8
24[1]	112	123	10.5	38.7	88	142.6	109.6	6.5
17-hydroxy-11-dehydrocorticosterone, 20 mg. 3 Dogs								
0	112	84	14.9	19.0	88	143.1	109.2	5.9
5	98	180	15.0					
8	98	178	15.2	19.0				
11	100	170	16.0	18.5	107	136.4	106.2	5.8
24[1]	112	102	14.5	21.3	114	142.4	110.2	4.5

[1] Dogs given food and water at 12th hour.

(5, 6, 14). While the explanation for this urea rise is not wholly clear, it is usually taken to be indicative of a faulty kidney function (6). Desoxycorticosterone acetate, which has a powerful action on the kidney, will correct the blood urea nitrogen changes in adrenal insufficiency. In fact, it will depress the urea levels below normal in both adrenalectomized and intact dogs (5, 9, 10). This depression could not be correlated with body fluid dilution or with blood sugar changes (5).

An appreciable rise in blood urea nitrogen in the untreated animals was found after only one of the four procedures, that of hemorrhage (table 4). Anuria followed all procedures, at least in the interval in which pressures were reduced well below normal; after hemorrhage, when blood volume as well as pressure was reduced, it was probably complete and sustained. The absence of electrolyte change in compound *E*-treated animals subjected to hemorrhage is confirmatory evidence. The urea rise after

hemorrhage is fairly extensive and rapid in both untreated and compound *E*-treated animals. Desoxycorticosterone acetate-treated dogs, however, showed but a minor change. The difference in action of the steroids is so striking, particularly in view of the apparent absence of renal function, that the explanation would seem to lie in an as yet undemonstrated depression of protein catabolism by desoxycorticosterone acetate.

TABLE 4. EFFECT OF ADRENAL HORMONES ON THE RESPONSE OF THE ADRENALECTOMIZED DOG SUBJECTED TO HEMORRHAGE

Hours	Blood Pressure, mm. Hg.	Pulse, /min.	Hemoglobin, gm. %	Blood Urea Nitrogen, mg. %	Blood Sugar, mg. %	Serum Sodium, m. eq./l.	Serum Chloride, m. eq./l.	Serum Potassium, m. eq./l.
Untreated. 3 Dogs; 16.6 cc. blood per kg. body weight withdrawn								
0	108	85	13.2	28.3	82	142.4	110.6	5.3
3	63	116	13.0					
5	52	122	11.4					
8	45	122	11.2	65.8	85	140.0	109.1	6.7
11	All dead or revived with cortical extract							
Desoxycorticosterone acetate, 20 mg. 4 Dogs on maintenance cortical extract; 32.7 cc. blood per kg. body weight withdrawn								
0	111	63	10.8	12.3	84	145.0	111.6	5.1
3	65	128	8.8					
5	70	130	7.9					
8	88	143	7.6					
11	90	140	7.0	17.7	84			
24	94	91	7.8	19.4	84	142.2	109.5	6.0
Desoxycorticosterone acetate, 20 mg. 4 Dogs on maintenance desoxycorticosterone acetate; 22.5 cc. blood per kg. body weight withdrawn								
0	120	82	12.8	16.9	90			
3	46	165	10.5		84			
5	52	170	10.4	24.8	85			
8	3 of 4 dead, other recovered by 24th hour							
17-hydroxy-11-dehydrocorticosterone, 20 mg. 3 Dogs; 23.0 cc. blood per kg. body weight withdrawn								
0	107	108	12.4	24.2	77	142.9	110.6	5.7
3	64	141	12.2					
5	68	136	11.2					
8	66	135	10.9					
11	63	133	10.5	49.7	90			
24	86	113	9.7	53.8	90	143.3	112.2	4.6

Two of the three corticosterone-treated animals subjected to visceral trauma showed an extensive and unexpected rise in blood urea nitrogen levels (table 1). The reason underlying this rise is not known. Minor variations between animals of a group and between groups of animals were encountered, but they are regarded as individual variation without clear significance.

Serum sodium and chloride. While in adrenal insufficiency there is an appreciable decline in serum sodium and chloride concentrations, there is no consistent and significant change in serum electrolyte accompanying any of the states of circulatory collapse except a small decline following the single stage bilateral adrenalectomy, (table 2). Cortical extract may or may not prevent this decline, depending on the

dosage employed. It would seem that more extract is required for this purpose than that essential for protection against circulatory failure. The thorough infiltration of the area surrounding the adrenal glands with procaine at the time of removal, which protected against cirulatory failure, also prevented the serum sodium and chloride concentration change. This would indicate that the fall in serum concentrations was associated in some way with derangements directly involved in the circulatory failure itself. Yet the fact that animals treated with desoxycorticosterone acetate showed no sodium and chloride change even at death indicates that the electrolyte loss does not play an essential rôle in the progressive development of the circulatory failure.

With the exception of hemorrhage, all procedures elicited a fall in serum sodium and chloride concentrations in the corticosterone and compound *E*-treated animals. This is surprising in view of the fact that untreated animals showed no comparable change, indicating that these steroids were actually favoring the loss of electrolyte from the blood. This action is explicable for compound *E* by the renal outpouring of sodium and chloride which has been reported to follow its administration (11). While the total decline in serum electrolyte shown by corticosterone and compound *E*-treated dogs was similar, a significant difference in the time interval at which these changes appeared is to be observed. Corticosterone-treated animals showed no change in serum concentrations until after the 12th hour; by this time the blood sugar, which had been elevated above normal, often began to decline, which might be taken as evidence that the available supply of corticosterone had been depleted. In the compound *E*-treated animals, however, the electrolyte decline was already well in evidence by the 12th hour. These dogs usually showed no symptoms, and the blood sugar did not begin to decline until the 48th to 72nd hour. Adequate amounts of the steroid must have been present at the termination of the experiment, indicating again that compound E was actually favoring the electrolyte loss.

Serum potassium. Adrenal insufficiency is usually accompanied by a rise in serum potassium concentration; in fact, the symptoms of insufficiency have been ascribed to potassium toxicity (12). Changes in potassium concentrations in these conditions of circulatory collapse have proved extremely variable, not only between groups but between animals of a group, so that definite conclusions can not be reached. The single-stage bilateral adrenalectomy and hemorrhage were usually associated with a rise in potassium, visceral trauma with a fall. A significant rise was often observed after muscle trauma (2–5 m. eq. per l). Any correlation between the type of therapy used and the degree of potassium change is quite obscure. Compound *E*-treated dogs often did show a decline where a rise was to be expected. The potassium changes were rarely of an order of magnitude which would justify attributing to them a causative rôle in the circulatory collapse.

Blood sugar. Of the four stress procedures employed, a fall in blood sugar was often observed following all but hemorrhage. After the single-stage bilateral adrenalectomy, the fall was quite usual, rapid and severe; after muscle trauma and intestinal stripping it was more inconstant and less extensive (tables 1–3). A part of the variation between animals seemingly can be attributed to the maintenance therapy used prior to the experiment. For example, animals which had been maintained on desoxycorticosterone acetate and then, after priming treatment with large amounts of desoxycorticosterone acetate, subjected to muscle trauma (table 3), always showed a decline in blood sugar. Animals maintained on cortical extract before the pre-treatment with desoxycorticosterone acetate either showed a slight decline or maintained normal sugar levels. It would seem that the presence of very small amounts of cortical

extract, quite insufficient to prevent circulatory failure, were still capable of maintaining normal sugar levels. Desoxycorticosterone acetate itself had no action in preventing the decline in blood sugar. Both corticosterone and compound *E* not only prevented the decline, but produced an actual elevation above normal. These sugar changes agree closely with those shown by Selye and Dosne (13) to follow visceral trauma in adrenalectomized rats treated with cortical extract. These authors link the sugar rise to the increased amounts of lactic acid which follow trauma. Compound *E* did not elevate the blood sugar after hemorrhage, nor was there a tendency for the blood sugar to decline in desoxycorticosterone acetate-treated and untreated animals following hemorrhage.

The significance of the decline in blood sugar following traumatic procedures in the adrenalectomized dog is not clear, for it cannot be directly correlated with changes in blood pressure (3). Nor can the symptoms of circulatory collapse be regarded in any way as due to hypoglycemia. The blood sugar change, however, should be considered as indicative of fundamental alterations in carbohydrate metabolism (15).

DISCUSSION

The blood studies reported emphasize the strong possibility of a connection between the protective action of adrenal steroids against circulatory failure and the influence they exert on carbohydrate metabolism. The circulatory failure following single-stage bilateral adrenalectomy, intestinal stripping, or muscle trauma is often associated with a decline in blood sugar. Desoxycorticosterone acetate, which has no or only partial effectiveness in protecting the circulation after these procedures, can not prevent this blood sugar change. Corticosterone and compound *E* not only prevent the decline but elevate the blood sugar above normal. At the same time they prevent circulatory failure. It has been suggested that the fault in carbohydrate metabolism involved might encompass derangements in the tissues of the vascular peripheral apparatus itself, so that their normal function could not be sustained in face of a severe stress.

Compound *E* is certainly less effective in protecting against the circulatory collapse which follows hemorrhage than against those following the other stress procedures. Several lines of evidence point toward the conclusion that hemorrhage is unique among the stress procedures employed. It does not involve appreciable trauma to nervous and non-nervous tissue, and does not lead to a decline in blood sugar. Compound *E* will not evoke the characteristic rise in sugar, which follows the other procedures.

Hemoconcentration is found after traumatic procedures which lead to an extensive local loss of fluid. After hemorrhage, however, there is an appreciable hemodilution, which is more rapid in onset and more extensive after desoxycorticosterone acetate treatment than with compound *E* therapy. Compound *E*-treated dogs react, in fact, much as do untreated animals. The character of the hemodilution and blood pressure changes indicates a vasoconstriction in the animals treated with desoxycorticosterone acetate greater than that observed in compound *E* and untreated dogs. Circulatory failure following hemorrhage is not to be correlated with the ability of the animal to show hemodilution, however, for even large amounts of desoxycorticosterone acetate will not prevent a rapid fall in blood pressure to the death level unless small amounts of carbohydrate active steroids contained in the cortical extract previously given for maintenance, are present. When these steroids are absent, the initial vasoconstriction of the desoxycorticosterone acetate-treated dogs cannot be sustained, and even precipitates the circulatory collapse.

Blood urea-nitrogen levels tend to show a much greater rise after hemorrhage than after the traumatic procedures. This urea rise is to be attributed in part to an interruption of normal renal function. Desoxycorticosterone acetate will prevent the rise whereas the other steroids are without effect. It is difficult to attribute the lack of appreciable change in urea levels in dogs treated with desoxycorticosterone acetate solely to an increased renal function. It seems probable that direct depression of protein catabolism by this steroid occurs. Since urea nitrogen changes can not be correlated with blood pressure changes, their significance is not clear.

There is certainly no evidence that the failure of the circulation can be related to electrolyte disturbances, for serum sodium and chloride concentrations are well-maintained after all procedures other than the single-stage bilateral adrenalectomy. Even here the decline is but slight, and can be fully corrected by desoxycorticosterone acetate even though this steroid will not prevent circulatory collapse.

SUMMARY

1. Blood analyses from a series of adrenalectomized dogs subjected to stress and treated with cortical extract, desoxycorticosterone, corticosterone, and 17-hydroxy-11-dehydrocorticosterone have been compiled.

2. The circulatory failure following stress procedures is accompanied by a hemoconcentration only when the traumatic procedure employed has led to a local loss of fluid. Desoxycorticosterone may have a slight effect in relieving the hemoconcentration following muscle trauma. The hemodilution which follows hemorrhage is more rapid in onset and extensive in desoxycorticosterone-treated animals than in 17-hydroxy-11-dehydrocorticosterone-treated and untreated dogs.

3. No appreciable rise in blood urea-nitrogen level followed the traumatic procedures, but urea changes did follow hemorrhage. This change could be corrected by desoxycorticosterone but not by 17-hydroxy-11-dehydrocorticosterone.

4. A decline in serum sodium and chloride concentrations was observed only after the single-stage bilateral adrenalectomy. While it could not prevent the circulatory failure which followed, desoxycorticosterone did prevent this change in serum electrolyte concentrations. The use of 17-hydroxy-11-dehydrocorticosterone was followed by significant declines in serum sodium and chloride concentrations. This appears confirmatory evidence for the finding that this steroid actually increases the renal excretion of these electrolytes. Serum potassium concentration changes in circulatory failure after the various stress procedures have proved extremely variable and rarely extensive.

5. A significant decline in blood sugar followed each of the traumatic procedures. There was no sugar change after hemorrhage. Corticosterone and 17-hydroxy-11-dehydrocorticosterone prevented this sugar change whereas desoxycorticosterone did not.

6. The ability of desoxycorticosterone to protect against circulatory collapse following hemorrhage is largely dependent upon the presence of some factor contained in the cortical extract used for maintenance prior to the experiment.

7. None of the blood chemistry findings reported give a direct explanation of the underlying deficiencies concerned in the circulatory collapse which follows the stress procedures. Several lines of evidence, including changes in blood sugar, indicate that a deep-seated disturbance in carbohydrate metabolism affecting the functional capacity of the tissues of the body may be present. Since such changes presumably affect the peripheral vasculature, they would be conducive to circulatory failure.

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ACTION OF VASOPRESSIN AND OXYTOCIN IN CAUSING PREMATURE OVIPOSITION BY DOMESTIC FOWL

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IN A PREVIOUS INVESTIGATION of the effect of pituitrin in causing the premature expulsion of avian eggs, a reaction first described by Riddle (1) in 1921, Burrows and Byerly (2, 3) found that eggs up to 20 hours of prematurity could be obtained from the domestic fowl within a few minutes following injection. In the present study the effects of preparations of relatively purified oxytocin and vasopressin on premature oviposition by the hen have been determined. The results indicate that premature oviposition can be effected by vasopressin alone, but that oxytocin when present in sufficiently high concentrations may also contribute to the reaction.

PROCEDURES

White Leghorn hens in their first year of production and Rhode Island Red hens in their second year of production were used in about equal numbers. The hens were kept in laying batteries, and hourly laying records were maintained regularly from 8:00 AM. to 4:00 P.M. By referring to these records, the time of expected oviposition could be estimated to within an hour. With the exception of a few hens used in preliminary experiments, only birds known to possess hard-shelled eggs in their uteri were injected. Injections were made from one hour or less to 6 hours before the expected time of lay, with the exception of only a few of the birds used in preliminary experiments. All injections were made intravenously.

Pitressin and pitocin[1] were used as sources of relatively purified vasopressin and oxytocin. Pitressin is assayed to contain 20 vasopressor units per cc. and is stated to contain not more than 0.05 unit of oxytocin per unit of vasopressin. Pitocin is standardized at 10 oxytocic units per cc. and contains not more than 0.05 vasopressor unit per unit of oxytocin. In all calculations of contaminant hormone in either pitressin or pitocin the maximal value, 5 per cent contamination, has been used as a reasonable basis for the evaluation of results (4).

In vitro reactions of the uterus to pitressin and pitocin were determined by immersing uterine strips in Ringer-Locke's solution and obtaining kymograph records of uterine contractions before and following the addition of the pituitary extracts to the perfusion fluid.

RESULTS

A number of preliminary experiments served to determine roughly the order of response, as measured by percentage yield of premature hard and soft shelled eggs, to injections of pitocin and pitressin. The results indicated that premature oviposition was brought about mainly by vasopressin, whether this was administered as pitressin or as a contaminant in pitocin.

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[1] The pitressin and pitocin were supplied by Parke, Davis and Co., Detroit, Mich.

On the basis of these preliminary and approximate findings, groups of similar hens were injected with pitressin and pitocin at doses calculated to be equivalent in content of vasopressin. With the exception of one group of injections at full strength, pitressin was diluted to 40 times its volume with normal saline in order that the same quantity of fluid might be injected at equivalent vasopressor doses. All injections were made within a period of 2 weeks.

The results of injections of pitressin and pitocin at equivalent vasopressor levels over the range 0.03125 to 0.25 units are given in table 1. In figure 1 the percentage yields recorded in table 1 are plotted against the logarithm of vasopressor units injected as pitressin and in pitocin. Circles indicate responses to pitressin, discs to pitocin. If we restrict comparisons to yields between 30.0 and 90.9 per cent in the case of pitressin and 28.0 to 89.2 per cent for pitocin the data satisfy reasonably well the relation $y = a + b \log x$, where y is percentage yield, x is the dose, and a and b are constants.

The straight line proportionality between yield and the logarithm of dose would not ordinarily be expected to hold for very high or very low yields, as Emmens (5) has recently emphasized in connection with other data. This usual limitation, taken together with the very small numbers of eggs obtained in excluded groups at low doses,

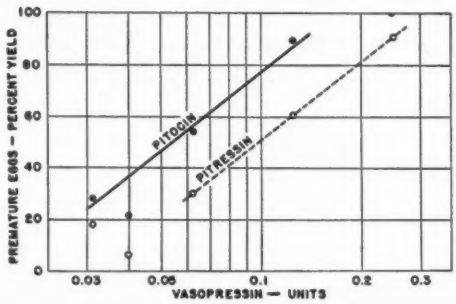

FIG. 1. PERCENTAGE YIELDS OF PREMATURE EGGS PLOTTED AGAINST VASOPRESSIN DOSES (LOGARITHMIC SCALE) ADMINISTERED AS PITOCIN AND PITRESSIN.

suggests that the straight line yield/logarithm dose curves of figure 1 afford a fair basis for evaluating the relative effects of pitressin and pitocin.

These curves show the ratio of vasopressin contents in pitressin and pitocin (vasopressin in pitressin:vasopressin in pitocin) to be approximately 2 at levels of equal yields. Recalling the labelled potencies and the assumed composition of the two preparations, it follows that for equal yields 0.05 cc. of pitressin is approximately equivalent to 1.00 cc. of pitocin, or 0.1 unit of vasopressin +0.005 unit of oxytocin are approximately equivalent to 0.05 unit of vasopressin +1.00 unit of oxytocin.

According to the second expression the amount of oxytocin in pitocin is 200-fold the amount in pitressin at levels of equivalent yield, and the vasopressin content of pitocin is one-half that of pitressin. It seems probable then that the oxytocin content of pitressin at effective levels of pitressin is below the threshold of effect.

The last column of table 1 records the average time required for expulsion of eggs following injection. In general, the higher doses of either pitocin or pitressin effect oviposition more promptly than do lower doses.

Pitressin and pitocin both caused severe contractions when administered in relatively large doses to perfused uterine strips and milder contractions with smaller

doses. The order of effectiveness of the two preparations appeared to be approximately the same as was their effectiveness in causing premature oviposition. McKenney et al. (6) have previously reported qualitatively similar reactions of excised uterine strips from fowls to pituitrin.

DISCUSSION

In stating the result that 0.1 unit of vasopressin +0.005 unit of oxytocin is approximately equivalent to 0.05 unit of vasopressin +1.00 unit of oxytocin in effecting the premature expulsion of hard-shelled eggs over the range of yield of 30 to 90 per cent, the content of contaminant hormone in pitocin and in pitressin is taken at 5 per cent. This is, however, a maximal value, and if the actual content of vasopressin in

TABLE 1. ACTION OF PITRESSIN AND PITOCIN INJECTED AT EQUIVALENT VASOPRESSOR LEVELS ON PREMATURE OVIPOSITION OF HARD-SHELLED EGGS

Preparation	Units of—		Number of Hens Injected	Premature Eggs		
	Vasopressin[1]	Oxytocin[1]		Number	Per cent	Reaction time, min.
Pitressin[2]	0.0313	0.0016	11	2	18.2	18.0
Pitocin		0.625	25	7	28.0	5.9
Pitressin	0.04	0.002	16	1	6.25	28.0
Pitocin		0.80	14	3	21.4	5.0
Pitressin	0.0625	0.0031	40	12	30.0	8.0
Pitocin		1.25	39	21	53.8	6.1
Pitressin	0.125	0.0063	38	23	60.5	4.1
Pitocin		2.50	37	33	89.2	3.5
Pitressin	0.25	0.0125	11	10	90.9	3.0
Pitocin		5.0	11	11	100.	4.5

[1] The oxytocin content of pitressin and the vasopressin content of pitocin were calculated on the assumption that pitocin and pitressin each carried 5 per cent of the contaminant hormone.
[2] Injected full strength with microsyringe.

pitocin were lower than 5 per cent the contribution of vasopressin administered in pitocin would be proportionally less, that of oxytocin proportionally higher, indicating possibly an additive effect of the two posterior pituitary hormones in causing premature oviposition. It is perhaps of interest in this connection that injections of pitocin in doses which did not cause premature oviposition often caused bodily straining apparently as severe as did injections of pitressin which effected oviposition.

Since there is no absolute unit of either vasopressin or oxytocin, differing responses may well lead to divergent results in comparing solutions varying greatly in indicated ratios of the two substances. Coon (7) has reported the assay for oxytocic substances by the depressor response of the fowl to yield higher values than does the official method when the vasopressin-oxytocin ratio is above 2.5. Gardiner and Bradbury (8) have shown that equivalent dilutions of pitocin and pitressin produced equivalent oxytocic effects in the intact human postpartum uterus "in spite of the wide variation in oxytocic values as assayed on the strips from the uterus of the virgin guinea pig" and concluded that present methods of assay are not an index of actual clinical oxytocic potency of pitressin. In view of the unsatisfactory status of both vasopressor and oxytocic units, the possibility that premature oviposition by the hen may be due entirely to an effect of vasopressin, an oxytocic activity not measured by

present methods of assay, must be recognized; it is equally possible that the contribution of oxytocin in pitocin may be larger than appears in terms of standard measures of potencies.

SUMMARY

Premature oviposition of hard-shelled eggs is effected in the hen by intravenous injection of pitressin or pitocin. Pitressin in doses of 0.0625 to 0.25 vasopressor units and pitocin in doses of 0.625 to 2.50 oxytocic units give yields of approximately 30 to 90 per cent. Percentage yields over these ranges following injections of pitressin and pitocin are proportional to the logarithm of vasopressin content, although equivalent yields require slightly less than twice the dose in pitressin as in pitocin.

In terms of the commonly assumed composition of pitressin and pitocin, 0.1 unit of vasopressin +0.005 unit of oxytocin is approximately equivalent to 0.05 unit of vasopressin +1.00 unit of oxytocin. Premature oviposition can apparently be effected by vasopressin alone, but oxytocin in sufficiently high concentrations appears also to contribute to the reaction.

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PROGESTERONE-LIKE ACTIVITY OF SOME STEROID COMPOUNDS AND OF DIETHYLSTILBESTROL IN STIMULATING MAMMARY LOBULE-ALVEOLAR GROWTH[1,2]

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FOLLOWING THE PURIFICATION, isolation and synthesis of progesterone, the hormone of the corpus luteum, it was shown that progesterone stimulated progestational proliferation of the uterus, induced sexual receptivity in the guinea pig and stimulated growth of the mammary lobule-alveolar system. For some time it was thought that progesterone was specific for these functions, but now numerous other compounds are known to have these same functions but to a lesser degree than progesterone.

Both testosterone and methyl testosterone have been shown to have slight progesterone-like activity by Klein and Parkes (1). Emmens and Parkes (2) reported that a new synthetic hormone, anhydro-hydroxy-progesterone (ethinyl testosterone or pregneninolone) possess about one-tenth the activity of injected progesterone in promoting progestational proliferation, and that it was equally potent by mouth or by injection. An adrenal cortical-like compound, desoxycorticosterone and the acetate also have been shown to promote progestational proliferation by Van Heuverswyn, Collins, Williams and Gardner (3) and by Leathem and Crafts (4).

Hertz, Meyer and Spielman (5) have reported on the specificity of progesterone in inducing sexual receptivity in the ovariectomized guinea pig. On the other hand Van Heuverswyn et al. (3) found that desoxycorticosterone has one-sixth to one-tenth the activity of progesterone in inducing sexual receptivity in the guinea pig while Torstveit and Mellish (6) similarly have reported that an aqueous extract of the adrenal cortex which contained the life-maintaining factor also produced the copulatory reflex in the guinea pig.

The growth of the mammary gland is also known to occur under the stimulation of the female sex hormones. Turner and Frank (7, 8) demonstrated in the rabbit that estrogen alone would stimulate only the duct system while a combination of estrogen and progestin was necessary to secure complete mammary gland growth. This has also been shown by Turner and Shultze (9) in the rat, and by Turner and Gomez (10) in the mouse. More recently Lyons and McGinty (11), Sharf and Lyons (12) with the rabbit and Mixner and Turner (13) with the mouse, have demonstrated more of the quantitative relationship existing between estrogen and progesterone in stimulating mammary gland growth.

Gardner (14) reported that slight growth of the mammary glands of hypophysectomized male mice was secured with the injection of desoxycorticosterone acetate, progesterone and estradiol dipropionate and combinations of these hormones. Also Mixner and Turner (15) secured growth of the mammary lobule-alveolar system of castrate female mice with pregneninolone, a synthetic progesterone-like compound. Reece and Mixner (16) reported that

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testosterone proprionate stimulated an extensive development of the lobule-alveolar system of the mammary glands of ovariectomized female rats. Diethylstilbestrol, a synthetic estrogenic compound, was reported to have mammary lobule-alveolar proliferating properties by Dodds, Lawson and Noble (17) in the guinea pig and by Lewis and Turner (18) in the mouse, rabbit and goat.

The object of this paper is to report the results of a study of the relative potencies of progesterone and certain other sterols in their ability to stimulate the lobule-alveolar system of the mammary glands in the ovariectomized female mouse.

PROCEDURE

The various compounds[3] were assayed for their mammary lobule-alveolar growth promoting properties by a technic recently developed by the writers (19). The crite

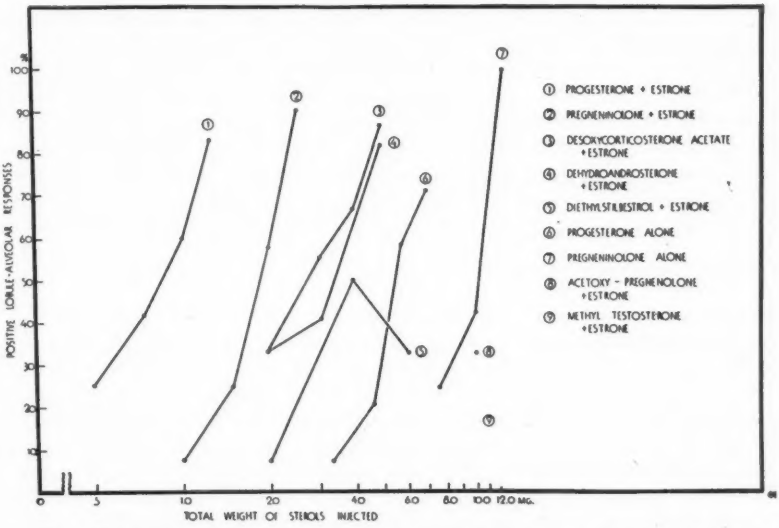

Fig. 1. Comparison of progesterone and other sterols in their ability to stimulate the lobule alveolar system of the mammary gland of the ovariectomized female mouse, plotted on arithlog paper. To secure a unit response ($50 \pm 10\%$) the following compounds with esterone required respectively, progesterone, 1 mg.; pregneninolone, 2 mg.; desoxycorticosterone acetate, 3 mg.; dehydroandrosterone, 3 mg.; and diethylstilbestrol, 4 mg. Without estrone, progesterone and pregneninolone required respectively 6 and 10 mg.

rion of response was the percentage of ovariectomized virgin female mice showing a minimum amount of lobule-alveolar growth after 10 daily subcutaneous injections of the material to be assayed. Estrone was injected with these various compounds for the reason that estrogen has been shown to enhance the activity of progesterone in stimulating mammary lobule-alveolar growth (13). Olive oil was used as the carrier for the compounds.

RESULTS

The group of compounds which were assayed for their mammary lobule-alveolar growth-promoting property include desoxycorticosterone acetate, dehydroandroster-

[3] The steroid compounds were supplied through the courtesy of Dr. E. Schwenk, of the Schering Corp., Bloomfield, N. J.

one, diethylstilbestrol, acetoxy-pregnenolone, methyl testosterone, testosterone and testosterone propionate (see table 1). These data which have been secured to date on all of these various compounds together with that for progesterone and pregneninolone are presented graphically on arithlog paper in figure 1. This figure shows *a*), that there are uniform increases in response to increasing dosages of the materials and *b*), that comparative potencies of the materials may be determined by reference to the weight scale of materials injected.

Progesterone (13) and pregneninolone (15) each with and without estrone have been assayed and reported previously. One mg. of progesterone and 2 mg. of pregneninolone each together with estrone were required to secure unit responses ($50 \pm 10\%$ positive). Progesterone is the most potent substance that has been assayed to date for

TABLE 1. LOBULE-ALVEOLAR RESPONSES OF CASTRATE FEMALE MICE TO SOME STEROID COMPOUNDS AND TO DIETHYLSTILBESTROL

Number of Mice	Compound		Estrone Dosage	Lobule-alveolar Responses		Positive Responses
	Name	Total Dosage		+	−	
		mg.	*μg. total*			%
12	Desoxycorticosterone acetate	2	7.5	4	8	33.3
9		3	7.5	5	4	55.6
12		4	7.5	8	4	66.7
7		5	7.5	6	1	85.7
12	Dehydroandrosterone	2	7.5	4	8	33.3
12		3	7.5	5	7	41.7
11		5	7.5	9	2	81.8
12	Diethylstilbestrol	2	7.5	1	11	8.3
12		4	6.0	6	6	50.0
12		6	9.0	4	8	33.3
8	Acetoxy-pregnenolone	5	7.5	0	8	0.0
12		10	7.5	4	8	33.3
7	Methyl testosterone	5	7.5	0	7	0.0
12		10	7.5	2	10	16.7
4	Testosterone	2	7.5	0	4	0.0
4		4	7.5	0	4	0.0
3		6	7.5	0	3	0.0
11		10	7.5	0	11	0.0
8	Testosterone propionate	5	7.5	0	8	0.0
6		10	7.5	0	6	0.0

its ability to stimulate mammary lobule-alveolar growth, followed by pregneninolone which possesses one-half the potency of progesterone according to the assay data on the two compounds.

As may be seen from table 1, both desoxycorticosterone acetate and dehydroandrosterone possess considerable mammary lobule-alveolar growth-promoting activity (with unit response at about 3 mg.), although the desoxycorticosterone appears to be slightly more potent. Thus on the basis of comparative assays these two compounds were about one third as potent as progesterone in stimulating mammary lobule-alveolar growth.

Diethylstilbestrol promoted mammary lobule-alveolar growth in these experiments, 4 mg. of this material being required for an assay response. This dose caused

only a slight weight decrease in the animals over the 10-day-assay period. A 6-mg. dose, however, severely decreased the body weights of the assay animals and this was reflected in the reduced response secured with the higher dosage. It thus appears that while diethylstilbestrol will stimulate lobule-alveolar growth in reasonably small doses, it is far more toxic at these levels than the sterols studied and therefore at dosages which should cause good lobule-alveolar growth the toxic properties predominate to the disadvantage of mammary gland growth.

Selye (20) reported on the hormonal activity of Δ^5-3 hydroxy-21 acetoxy-pregnene-20 one, or acetoxy-pregnenolone, an intermediary product in the Steiger and Richstein synthesis of desoxycorticosterone acetate. It possesses corticoid activity only slightly less than that of desoxycorticosterone acetate. In this study 10 mg. of this material with estrone caused 4 of 12 mice to respond with mammary lobule-alveolar growth. This would make it approximately one-sixteenth as potent as progesterone or one-fifth as potent as desoxycorticosterone acetate in stimulating mammary lobule-alveolar growth.

Ten mg. of methyl testosterone, an orally active androgen, caused 2 of 12 mice to respond with mammary lobule-alveolar growth indicating that it is approximately one twenty-fifth as active as progesterone in this respect.

Reece and Mixner (16) have shown that the injection of testosterone propionate into sexually mature spayed female rats caused an extensive development of the mammary lobule-alveolar system. However, in the present experiments neither testosterone or its propionate together with estrone caused any lobule-alveolar proliferation in castrate female mice when given in amounts up to 10 mg. during a 10-day period.

DISCUSSION

The activity of progesterone, pregneninolone, desoxycorticosterone acetate and methyl testosterone in promoting mammary lobule-alveolar growth as assayed and reported in this paper corresponds in a rough way to the activity of these substances in stimulating progestational proliferation of the uterus as reported in the literature. It appears that the early ideas on the specificity of progesterone must be revised as it is now apparent that numerous sterols have progesterone-like activity in varying degrees. Whether or not the naturally occurring sterols investigated, or others, actually play a progesterone-like rôle in the normal female is still an open question.

It is significant that there are a series of compounds which exhibit a property of progesterone, namely, that of stimulating mammary lobule-alveolar growth, but varying greatly in potency. A parallel might be drawn between this series and the estrogens. With the estrogens, a relatively inexpensive synthetic compound, diethylstilbestrol has been synthesized and found to have great estrogenic potency. It is not too much to hope that a comparable progesterone-like material also will be found that will have high activity and yet be relatively inexpensive.

The failure to secure lobule-alveolar growth with testosterone in these ovariectomized mice is interesting in view of the positive results secured by Reece and Mixner (16) in ovariectomized female rats. The mature male rat has a fair extension of the mammary duct system with lobule-alveolar development in contrast to the glands of male mice (of the same strain as the females used in these experiments) which have only rudimentary mammary glands. These glands of the male mouse are not sensitive to the stimulation of testosterone propionate (21). However, they are sensitive to the stimulation of dehydroandrosterone (21) and similarly the glands of female mice respond well to dehydroandrosterone with lobule-alveolar growth as may be seen from table 1. This discussion is intended to indicate that there are

definite species differences in the ability of both the male and the female to respond to certain of the male sex hormones with mammary gland growth. These differences probably explain the variation in the degree of development of the mammary gland of the males of the species. That there may be strain as well as species differences is indicated by the observation made in this laboratory that the males of a Swiss strain of mice secured from a commercial breeder had a high proportion of fairly well-developed mammary dusts. This is in contrast to the usual rudimentary mammary gland of the male mouse.

SUMMARY

Desoxycorticosterone acetate, dehydroandrosterone, diethylstilbestrol, acetoxy-pregnenolone, methyl testosterone, testosterone and testosterone propionate were assayed and compared with progesterone for their ability to stimulate mammary lobule-alveolar growth in the ovariectomized female mouse. Using progesterone as a standard, pregneninolone had an activity of approximately one-half, desoxycorticosterone acetate and dehydroandrosterone of one-third, diethylstilbestrol of one-fourth, acetoxy-pregnenolone of one-sixteenth, and methyl testosterone of one twenty-fifth, that of progesterone. Testosterone and testosterone propionate in doses up to 10 mg. failed to stimulate lobule-alveolar growth.

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STUDIES CONCERNING THE MECHANISM CONTROLLING THE INITIATION OF LACTATION AT PARTURITION[1,2]

I. CAN ESTROGEN SUPPRESS THE LACTOGENIC HORMONE OF THE PITUITARY?

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THE MECHANISM RESPONSIBLE for the control of the initiation of lactation at parturition has long remained a mystery. No definite progress was made on this problem until 1928 and 1929 when Stricker and Grueter (1, 2) announced the discovery of a factor in the pituitary which was capable of initiating lactation in animals whose mammary glands had been suitably developed. This work was soon confirmed in this country by Corner (3), Nelson and Pfiffner (4), Asdell (5), and Turner and Gardner (6). Riddle and his co-workers (7, 8) suggested the use of the pigeon as a sensitive assay animal, which has proven invaluable in the study of the chemistry and physiology of this hormone. Excellent extraction methods for obtaining lactogen in relatively pure form were devised by Bates and Riddle (9), Lyons (10), and Bergman and Turner (11). This laboratory has contributed to the knowledge of the relation of this hormone to other physiological processes by studying the content of lactogenic hormone in the pituitary as influenced by other hormones, growth, sex, species, pregnancy, lactation and nursing.

In a previous publication (12) the various theories which have sought to explain the absence of lactation during pregancy and its initiation post partum were reviewed. In these theories it was suggested that one or more agents suppressed milk secretion during pregnancy, but that at the time of parturition the inhibiting agent or agents were removed, permitting the onset of lactation. Undoubtedly the theory which seemed most plausible at the time and is still widely held was that advanced by Nelson (13), who suggested that the large amounts of estrogen known to be present during pregnancy suppressed lactation by inhibiting the secretion of lactogenic hormone in the pituitary and/or by a direct inhibitory effect on the mammary glands. As proof for this theory Nelson offered evidence that large dosages of estrogen can inhibit lactation, at least temporarily, in lactating guinea pigs. Other investigators have also claimed that estrogen, when administered in large quantities, can either diminish or completely suppress lactation in various animals: the mouse (14, 15, 16), the rat (17, 18, 19), the goat (20), and the cow (21, 22, 23). In addition, estrogens (particularly diethylstilbesterol) and testosterone propionate have been used extensively to inhibit lactation in postpartum women (24–33). However, a critical examination of the literature fails to support these claims in every case. Thus, in the rabbit (34) the injection of 4000 R.U. of estrogen for 6 days had no convincingly inhibitory effect on lactation, while in the castrate-lactating rat large amounts of estrogen were likewise reported to be ineffective in inhibiting lactation (35, 36). Furthermore, it is important to take into account the method of measuring

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the degree of lactation in these experimental animals. In the mouse and rat the growth rate of the young has been used as the criterion of lactation, but there is good reason to doubt that this is an accurate measure. Several investigators (23, 37) have shown that when large amounts of estrogen are administered to lactating rats, a considerable amount of this hormone may be excreted in the milk. In view of the deleterious effects of estrogens on growth (38–44) this factor alone could account for the decreased growth rate and death of the young. It is also possible that large dosages of estrogen may have injurious effects on the lactating mothers as well, and this seems likely from the finding that there is a decrease in weight of lactating mother-rats following estrogen treatment (19). Finally, as Turner (45) pointed out, "the injection of estrogen, producing an exceedingly nervous condition associated with the production of 'heat,' would interfere with the 'letting down' of milk and thus cause an apparent but not real suppression of milk secretion."

In the human the validity of the results obtained can also be questioned, since in almost all of these attempts to inhibit breast secretion with estrogens or androgens, the babies were removed from the breast simultaneously with the administration of hormones. Inasmuch as the maintenance of lactation has been demonstrated to depend on the nervous stimulus of suckling (46, 47) which maintains a high secretion of lactogenic hormone by the pituitary (48–50) it naturally follows that in the non-nursed breast, milk secretion will soon cease even in the absence of hormone treatment. In such cases it is difficult to determine just what additional effects can be attributed to large dosages of estrogens or androgens. Abarbanel et al. (51) overcame this objection by permitting parturient women to be nursed during the administration of diethylstilbestrol. He found, in a study involving more than 300 women, that diethylstilbestrol in dosages as high as 1.0 gm. failed to inhibit the onset of lactation in nursing women, while 50 to 500 mg. failed to inhibit established lactation in nursing women. Later, Abarbanel (52) showed that huge amounts of testosterone propionate, methyl testosterone, anhydro-oxy-progesterone and progesterone also failed to inhibit either the onset or the maintenance of lactation in women who were nursed.

The latter author pointed out that the relief of breast engorgement with large amounts of estrogens or androgens is not synonymous with inhibition of lactation. Painful breast engorgement is not due to a filling of the breast with milk, but to a lymphatic and venous stasis (53). Quoting from Abarbanel and Goodfriend (51): "Unfortunately, most of the clinical reports on the effects of estrogens upon lactation in the human being have confused painful engorgement with the onset of lactation, using these terms interchangeably. These are two distinct and separate phenomena."[3] This receives support from the clinical findings of a number of investigators (28, 30, 32, 54) who reported the relief of painful engorgement of the breast upon the administration of estrogens or androgens, while at the same time little or no inhibition of lactation was obtained. Finally, it may be stated that few investigators agree as to the degree of lactation suppression which can be obtained with estrogens. Milk secretion is often spontaneously re-initiated in women after the cessation of estrogen or androgen therapy (27, 28, 30, 32, 33, 55).

With the above considerations in mind, experiments were undertaken *a*), to determine whether large dosages of diethylstilbestrol or testosterone propionate would suppress either the lactogenic hormone of the pituitary or lactation in the postpartum rat; *b*), to determine the effects of the absence of nursing on the lactogenic hormone and lactation in parturient rats; and *c*), to determine the effects of increasing amounts of diethylstilbestrol on the lactogen content of normal male guinea pig pituitaries.

PROCEDURE AND RESULTS

Experiments with Lactating Rats

Twenty-four parturient albino rats, divided into four groups of 6 each, were used in the first experiments. Six rats served as controls and received no treatment; 6 were

[3] The reader is also referred to the discussion by Mixner and Turner of the relation of estrogen to the growth of the mammary gland and the production of hyperemia and capillary permeability in the stromal tissue of the gland (69).

injected subcutaneously with 2.00 mg. of diethylstilbestrol[4] daily (approximately equal to 100,000 I.U. of estrone daily) for the first 6 days post partum; 6 received 2.00 mg. of testosterone propionate[4] daily by subcutaneous injection for the first 6 days post partum; and 6 received no hormone and were never suckled by the young. In the case of this last group the litters were simply permitted to drop through a wide wire floor at the time of parturition. All of the mother rats were killed on the seventh day post partum, and the pituitaries and mammary glands were removed. The mammary glands were arbitrarily rated as + to ++++ by gross examination, depending on their degree of distension with milk. The size of each litter was determined on the

TABLE 1. EFFECT OF DIETHYLSTILBESTROL AND TESTOSTERONE PROPIONATE ON THE LACTOGEN CONTENT OF THE PITUITARY AND LACTATION IN RATS

Treated rats injected first 6 days post partum, all rats killed on 7th day

Number of Rats	Total Amount of Hormone Injected per Rat mg.	Average Body Weight gm.	Average Pituitary Weight mg.	Average Reece-Turner Units, per			Average % of Young Dead on 7th Day Post Partum	Average Rating of Mammary Secretions
				Pituitary	mg. Pituitary Tissue	100-gm. Body Weight		
6	Control lactators, no hormone	182	14.08	10.75	.76	5.9	0	++++
6	Diethylstilbestrol, 12 mg.	176	14.12	14.25	1.00	8.0	38	+++
6	Testosterone propionate, 12 mg.	202	13.46	12.25	.91	6.0	11	+++
6	No nursing, no hormone	217	10.13	5.10	.50	2.3		

day of birth, and on the seventh day after birth, at which time the stomachs of the young were observed for the presence of milk. The pituitaries of each group of mother rats were combined, macerated in a small agate mortar, suspended in a measured amount of distilled water, and assayed for lactogen by the Reece-Turner method (17). One Reece-Turner unit (R-T.U.) is equal to .045 internationl units of lactogenic hormone (56).

Results. As can be seen in table 1, the 6 control lactating rats contained an average of 10.75 R-T.U. or .48 I.U. of lactogen per pituitary on the seventh day after parturition. Their mammary glands were widely distended with milk (++++) and all of the young were alive. The six diethylstilbestrol-treated rats contained an average of 14.25 R-T.U. or .64 I.U. per pituitary which represents a 32.5 per cent increase in lactogenic hormone. The mammary glands of this group were less distended with milk (+++) than the control lactating rats, and 38 per cent of the young were dead by the seventh day. However, all of the living young contained milk in their stomachs. In the case of the rats treated with testosterone propionate their pituitaries assayed an average of 12.25 R-T.U. or .56 I.U. of lactogenic hormone, this also representing an increase rather than a decrease in lactogen. The mammary glands of this group contained about the same amount of milk (+++) as those of the diethylstilbestrol-treated animals, although only 11 per cent of the young had died by the seventh day post partum. The living young all contained milk in their stomachs. In the case of the 6 mother rats which were never suckled and received no hormone treatment, an average lactogen content of 5.10 R-T.U. or .23 I.U. per pituitary was obtained. This

[4] The testosterone propionate was supplied by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J., and the diethylstilbestrol by Dr. D. F. Green of Merck & Co., Rahway, N. J.

represents a decrease in lactogenic hormone of approximately 50 per cent as compared to the control lactators, and is practically as low as in the non-lactating estrous rat. Practically no milk was visible in the mammary glands of these rats on the seventh day after parturition.

These results show that high dosages of diethylstilbestrol or testosterone propionate will not inhibit the production of lactogenic hormone by the pituitary. How-

TABLE 2. EFFECT OF INCREASING DOSES OF DIETHYLSTILBESTROL ON THE LACTOGENIC CONTENT OF THE PITUITARY OF MALE GUINEA PIGS

Number of Guinea Pigs	Total Amount Stilbestrol Injected per Pig, mg.	Daily Amount Stilbestrol Injected, per Pig, mg.	Average Body Weight gm.	Average Pituitary Weight mg.	Average Reece-Turner Units, per			Average % Increase of Lactogen per 100 gm. Body Weight
					Pituitary	mg. of Pituitary	100 gm. Body Weight	
Male animals injected for 5-day period								
11	Controls		337	9.86	3.06	0.31	0.90	
5	0.0125	.0025	329	11.74	6.62	0.56	2.01	123
5	0.025	.0050	393	11.78	10.50	0.88	2.67	196
5	0.05	.01	317	13.86	14.25	1.02	4.49	398
5	0.10	.02	338	12.67	15.00	1.18	4.43	388
5	0.20	.04	340	13.03	16.50	1.26	4.85	438
5	0.30	.06	373	12.46	10.25	0.82	2.72	202
5	0.40	.08	303	12.87	8.37	0.65	2.75	205
5	1.00	.20	335	10.52	7.00	0.66	2.06	128
5	2.00	.40	322	12.10	7.50	0.62	2.01	122
5	10.00	2.00	334	11.01	8.12	0.73	2.43	170
5	50.00	10.00	322	10.94	5.37	0.49	1.66	84
Large male guinea pigs injected for 10-day period								
5	Controls	0.00	495	14.60	11.00	0.75	2.22	
5	20.00	2.00	523	14.46	14.25	0.98	2.72	22
5	100.00	10.00	510	14.73	14.75	1.00	2.89	30

ever, there was some reduction in milk secretion, as well as death of some of the young. It was also evident that the living young of the mothers treated with diethylstilbestrol and testosterone propionate were not as large on the seventh day following parturition as those in the control litters, although they all contained some milk in their stomachs. The low lactogen content which was found in the pituitaries of the mother rats which were not suckled explains why milk secretion had ceased in these animals. This further demonstrates that suckling is absolutely essential for the maintenance of lactation, since without the stimulus of nursing the high postpartum lactogen production of the pituitary is not maintained. This also explains why the results claimed with estrogens and androgens in inhibiting lactation in human beings are difficult to appraise correctly. It remains to be seen how much of such inhibition was due to the fact that breast nursing was discontinued and how much to hormone administration.

Experiments with Normal Male Guinea Pigs

In a further attempt to see whether estrogens can inhibit the production of lactogenic hormone by the pituitary, normal male guinea pigs were injected with increasing amounts of diethylstilbestrol. Eleven groups of small male guinea pigs were injected with total amounts of diethylstilbestrol ranging from .0125 to 50.00 mg. over a 5-day period and compared with a control group. Also two groups of large, mature male

guinea pigs, whose normal lactogen content per pituitary is more than 3 times as high as in the immature male guinea pigs, were injected with a total of 20.00 and 100.00 mg. of diethylstilbestrol respectively over a 10-day period in a further attempt to reduce the lactogen content of the pituitary. The pituitaries of each group of guinea pigs were assayed by the Reece-Turner method.

Results. It will be seen that a progressive increase was obtained in the lactogen content of the pituitary with .0125 to .05 mg. of diethylstilbestrol (table 2). Approximately the same increase in lactogen was secured with .05, .10 and .20 mg. of diethylstilbestrol (398 to 438 per cent). It is interesting that the 16.50 R-T.U. of lactogen

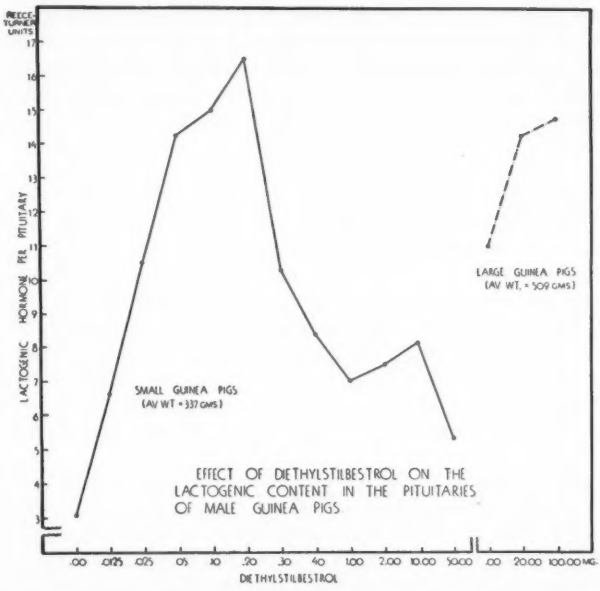
REECE-TURNER UNITS
LACTOGENIC HORMONE PER PITUITARY
SMALL GUINEA PIGS (AV WT = 337 GMS)
LARGE GUINEA PIGS (AV WT. = 509 GMS)
EFFECT OF DIETHYLSTILBESTROL ON THE LACTOGENIC CONTENT IN THE PITUITARIES OF MALE GUINEA PIGS
.00 .0125 .025 .05 .10 .20 .30 .40 1.00 2.00 10.00 50.00 .00 20.00 100.00 MG.
DIETHYLSTILBESTROL

Fig. 1. An optimum rise in the lactogen content of the pituitaries of small guinea pigs was obtained with 0.05 to 0.20 mg. of diethylstilbestrol; heavier doses of diethylstilbestrol resulted in smaller increases, although in no case was the lactogen content of the pituitary reduced below the normal level. Huge doses of diethyl stilbestrol administered to large guinea pigs also failed to effect any reduction in the initially high pituitary lactogen content, but actually elicited a small increase.

obtained in the group receiving .20 mg. of diethylstilbestrol is almost as high as the lactogenic hormone in the pituitaries of guinea pigs in full lactation (18.38 R-T.U.). The significance of this great rise in the lactogenic hormone of the pituitary which follows upon the administration of suitable doses of estrogen, will be fully discussed in the second and third paper of this series. It will be noticed that as the amounts of diethylstilbestrol were increased beyond the optimum levels of 0.05 to 0.20 mg., a steady decline resulted in the extent of increase of the lactogenic hormone. The highest dosage of diethylstilbestrol used, 50.00 mg. (approximately equal to 2,500,000 I.U. of estrone) gave the smallest increase in lactogen, 84 per cent (fig. 1).

In the case of the large guinea pigs, when an attempt was made to reduce the initially high lactogen content in the pituitary (11.00 R-T.U.) with huge dosages of diethylstilbestrol, no decrease was actually effected. In fact, the administration of 20.00 mg. of diethylstilbestrol (equal to 1,000,000 I.U. of estrone) brought a 22 per

cent increase (14.25 R-T.U.), whereas 100.00 mg. of diethylstilbestrol (equal to 5,000,000 I.U. of estrone) elicited a 30 per cent increase in the lactogen content of the pituitary (14.75 R-T.U.). It was considered futile to administer still higher dosages of diethylstilbestrol to guinea pigs, since these amounts are much in excess of the amounts used by other workers to inhibit lactation in laboratory animals. Even in the human being amounts of diethylstilbestrol smaller than 100.00 mg. have been reported to inhibit lactation (27, 28, 29, 55).

These results show that although smaller amounts of diethylstilbestrol can augment the lactogenic production in the pituitary to a greater extent than larger dosages, even the largest amounts of hormone injected did not decrease the amount of lactogen normally present in the pituitary. Therefore, any suppression or partial suppression of lactation which can be secured with estrogens is probably obtained through channels other than the suppression of the lactogenic hormone. There is not the slightest evidence available for the common assumption that estrogens inhibit lactation by suppressing the secretion of the lactogenic hormone of the pituitary.

DISCUSSION

The estrogen-inhibition theory advocated by Nelson (13) to explain the absence of copious lactation during pregnancy has been shown previously to be incorrect (12). It was found that rabbits which were simultaneously pregnant and lactating for 20 days (the does were re-bred on the day of parturition) contained just as much lactogenic hormone in their pituitaries and secreted just as much milk (as judged by the weight of the young) as rabbits which were lactating only. In short, these pregnant-lactating rabbits were presumably exposed to the same amount of estrogen as would be present during pregnancy alone, and yet there was no inhibitory effect on the lactogenic hormone or lactation. This must also be true for the mouse, rat, goat, cow and human being, since these species are also capable of being pregnant and lactating at the same time.

In no case has it ever been demonstrated that estrogens or androgens can actually decrease the secretion of lactogenic hormone by the pituitary. The present experiments show that large dosages of diethylstilbestrol and testosterone propionate do not decrease the lactogen content of the pituitaries of lactating rats but actually increase it. Similar results had been obtained previously by Reece and Turner (17). They found that six lactating rats, injected daily with 1000 I.U. of estrone for the first 7 days postpartum, also showed a small increase in the lactogen content of the pituitary (13.3 R-T.U. or .60 I.U.). There is ample evidence available that estrogens (17, 58, 60) and testosterone propionate (59) can greatly augment the lactogenic hormone of the pituitary. It must not be supposed that such increases in the lactogenic hormone of the pituitary merely represent a storage phenomenon. Recently it was possible to demonstrate, by assaying the lactogen content of the blood, that a simultaneous rise in the lactogenic hormone occurs in the bloodstream as well as in the pituitaries of male rabbits injected with estrone (60). In addition, a number of investigators have reported the initiation of lactation following the administration of estrogens (17, 23, 58, 61, 62).

The apparent inhibition of lactation with estrogens and the partial decrease in milk secretion which we found in our rats treated with diethylstilbestrol and testosterone propionate must be due to factors other than a decrease in lactogen secretion. It has been suggested (19, 63) that the decrease in lactation is due to the growth stimulus exerted by estrogen and pituitary mammogen I on the glandular epithelium of the mammary gland. It is assumed that a growing cell cannot secrete at the same time. There is no proof available that such an assumption is correct. It seems likely, however,

that in cases of simultaneous pregnancy and lactation, growth of the mammary glands and lactation occur together and that the administration of estrogens initiates lactation in some animals even as the mammary glands are growing. Finally, a report by Gardner (64) indicates that large amounts of estrogen may actually inhibit rather than favor mammary growth. It appears more probable that other factors are responsible for the apparent decrease or inhibition of lactation secured with estrogens.

The decrease in milk secretion which we noticed in the mammary glands of the rats treated with diethylstilbestrol and testosterone propionate may be due, in part, to a decreased secretion by the pituitary of hormones other than lactogen. Large amounts of estrogen have been shown to decrease the gonadotropic content of the pituitary (65–67). Inasmuch as hormones other than lactogen may be limiting factors in lactation, i.e. thyroxine and cortin, the temporarily diminished secretion of these other hormones might well produce a decline in lactation. Recently (68) it has been demonstrated that the adverse effects which can be secured with estrogens on body growth, can be remedied by the administration of anterior pituitary extracts. The assumption is that large amounts of estrogen inhibit the secretion of the hormone or hormones in the pituitary essential for growth. It would be interesting to determine whether the apparent inhibition of lactation secured in some animals upon estrogen treatment cannot also be overcome by the administration of anterior pituitary extracts.

SUMMARY AND CONCLUSIONS

1. The administration of large dosages of diethylstilbestrol (2 mg. daily) and testosterone propionate (2 mg. daily) to lactating mother rats for the first 6 days post partum *a*), did not decrease the content of lactogenic hormone in the pituitary, but actually increased it; *b*), caused some reduction in the amount of milk present in the mammary glands; and *c*), resulted in the death of 38 and 11 per cent respectively of the young rats.

2. The injection of total amounts of diethylstilbestrol ranging from .0125 to 100.00 mg. into normal immature and mature male guinea pigs did not reduce the lactogen content of the pituitary but actually increased it. Lower dosages of hormone resulted in greater increases in the lactogenic hormone (up to 438 per cent) than was secured with the higher dosages (22 to 84 per cent increase). It is concluded, therefore, that the administration of low, moderate, or huge amounts of estrogens will not produce a decrease in the secretion of lactogenic hormone by the pituitary.

3. Parturient rats which were not suckled for the first week after parturition contained 50 per cent less lactogen in their pituitaries than suckled rats, and their mammary glands were practically devoid of milk. This explains why lactation will cease when suckling is discontinued. It also explains why the clinical reports on the suppression of lactation with hormones are difficult to evaluate.

4. It is suggested that the apparent decrease in lactation which has been reported in some animals with large injections of hormone, and which was secured in our rats treated with diethylstilbestrol and testosterone propionate is probably due to a decrease in secretion of anterior pituitary hormones other than lactogen. These anterior pituitary hormones are believed to influence the yield of milk through their action in depressing the availability of the precursors of milk in the blood rather than the potential function or capacity of the mammary epithelium to secrete milk.

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STUDIES CONCERNING THE MECHANISM CONTROLLING THE INITIATION OF LACTATION AT PARTURITION[1,2]

II. WHY LACTATION IS NOT INITIATED DURING PREGNANCY

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LACTATION IS NOT INITIATED during pregnancy principally because the amount of lactogenic hormone produced by the pituitary is too low to support lactation. Thus in the rat (1) and guinea pig (2) the lactogen content of the pituitary is no higher during pregnancy than in the nonpregnant state. The same is true for the pregnant rabbit, although a slight rise in the lactogenic hormone occurs just a few days prior to parturition (2). Within two to five days post partum, however, there is a 200 to more than 400 per cent increase in the lactogenic hormone of the pituitaries of these animals. Also in parturient women, the amount of lactogenic hormone found in the urine is 8 to 16 times as much as can be obtained during pregnancy (3). It is the object of this paper to determine, if possible, why the secretion of lactogenic hormone remains at a low level during pregnancy.

In the previous paper (4) it was demonstrated that small, moderate or huge dosages of diethylstilbestrol will not inhibit the production of lactogenic hormone by the pituitary, but will actually increase it. With one exception, all investigators who have studied the effects of estrogen on the lactogen content of the pituitary agree that lactogen production is enhanced thereby. Simpson and Evans (5) have reported that estrogen-treated rats contain a decreased amount of lactogen in their pituitaries. Somewhat later, however, the California workers in citing their earlier work claimed that they actually obtained an increase in the lactogen content of the rat pituitary (6).

Reece and Turner (1) were the first to report that the injection of estrone or estradiol would increase the lactogen content in the pituitaries of male and female rats, and male guinea pigs. Testosterone propionate (7) and diethylstilbestrol (4, 8) were likewise shown to possess these properties. Administration of the former hormone elicited an increase of 40 per cent in the lactogen content of the pituitaries of mature spayed rats, while the latter hormone produced a 226 per cent increase in the pituitaries of mature spayed rats and a 438 per cent increase in the pituitaries of immature male guinea pigs. The increased lactogen content of the pituitary which the present authors obtained with estrone and diethylstilbestrol in the case of the immature guinea pig (17.75 and 16.50 R.T.U.[3] respectively) is practically equal to the greatest amount of lactogen (18.38 R.T.U.) found in the pituitaries of parturient lactating guinea pigs (2). During the estrous cycle of the rat the lactogen content of the pituitary was found to be greatest during proestrum and estrum, when estrogen

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[3] Reece-Turner units of lactogenic hormone (1). One R-T. U. = .045 international units (57).

is most active, and lowest during diestrum and metestrum (1). The lactogen content of the guinea pig pituitary is also higher during estrum than diestrum (9). After ovariectomy in the rat and following castration in bulls, there was a marked reduction in the lactogen content of the pituitary (1). In growing animals, there is a significant rise in the lactogen content of the pituitary of the female rat (1), female rabbit, and male and female guinea pigs (10) at the time of puberty. All these experiments offer convincing proof that the production of lactogenic hormone by the pituitary can be markedly stimulated by estrogens (or androgens).

In addition to the increase in the lactogen content of the pituitary which can be secured with estrogens and androgens, a number of investigators have reported the actual initiation of lactation with these hormones. Milk secretion in rats was initiated following the administration of estradiol (1), testosterone propionate (7), and diethylstilbestrol (8). Diethylstilbestrol has also initiated lactation in rabbits (11), goats (12, 13) and cows (14). Folley et al. (12) obtained a maximum yield of 1500 cc. of milk daily from each of three virgin goats treated with moderate amounts of diethylstilbestrol dipropionate, while Lewis and Turner (13) reported the induction of lactation in kids as well as in virgin goats following and during the administration of diethylstilbestrol. Walker and Stanley (14) have recently found that a considerable milk yield could also be obtained from a castrate and a sterile cow following and during treatment with diethylstilbestrol dipropionate. A maximum of 16 pounds of milk daily was obtained from the former animal while as much as 14 pounds was secured from the latter animal. It is interesting that these authors reported an actual increase in milk production during the period of administration of moderate dosages of diethylstilbestrol. No inhibition of lactation was obtained until much higher dosages of hormone were given.

In addition, proof has been obtained indicating that the increased lactogen content of the pituitary which can be secured with estrogen is coincident with an increased release of lactogen into the blood stream (15). Three groups of male rabbits were injected with three different levels of estrone over a 10-day period. Assays of the pituitaries and blood of these rabbits revealed that a simultaneous increase in lactogen was obtained in both the pituitary and blood-stream.

In view of all the foregoing evidence that estrogen can increase the production of lactogenic hormone by the pituitary and initiate milk secretion, it was difficult to understand why, during pregnancy, when large amounts of estrogen are known to be present, there was no rise in the lactogenic hormone of the pituitary. It would appear that some factor must be present during pregnancy which overrides and nullifies the stimulating effect of estrogen on the lactogenic hormone. It seemed likely that this factor was progesterone, inasmuch as large amounts of this hormone, as well as estrogen, are present during pregnancy. Furthermore, there is ample evidence available which indicates that during pregnancy progesterone is predominant and overrides certain physiological effects normally produced by estrogen. Experimentally, it has been demonstrated that, provided the proper dosage ratios obtain, progesterone can prevent the normal effects of estrogen in producing *a*), estrus in rats (16, 17); *b*), the sexual skin reaction in monkeys (18); *c*), an increase in uterine motility (19) and *d*), an increase in the size of the pituitary (20). Contrawise, and also under proper quantitative relationships, estrogen has been shown to nullify the effects of progesterone in producing *a*), progestational proliferation in the rabbit uterus (21–24); *b*), placentomata (25) and *c*), growth of the mammary lobule-alveolar system (26).

In connection with the problem of the mechanism controlling the initiation of lactation at parturition, it was our purpose to determine experimentally whether progesterone, in addition to having the effects on estrogen already enumerated, would also override the effect of estrogen in increasing the content of lactogenic hormone in the pituitary.

EXPERIMENTAL PROCEDURE

Immature female guinea pigs, weighing approximately 300 gm., were used in this experiment. Ten animals served as controls and received no hormone, while 8 animals received progesterone only. All of the remaining guinea pigs were paired into groups

of five each, one group receiving estrone alone and the other a combination of estrone and progesterone.[4] The amount of progesterone was always held constant at one mg. per day and only the dosage of estrone was varied. All injections were made over a 5-day period, and the animals were autopsied on the sixth day. The pituitaries of the estrone-treated guinea pigs were compared with those of the estrone-progesterone-treated animals by the Reece-Turner pigeon method (1). As a further check on the action of estrone alone and of estrone in combination with progesterone, the number

TABLE 1. EFFECT OF ESTRONE AND ESTRONE-PROGESTERONE COMBINATIONS ON LACTOGEN CONTENT IN THE ANTERIOR PITUITARY OF IMMATURE FEMALE GUINEA PIGS

All animals injected for 5-day period

Number of Guinea Pigs	Total Dosage Hormone Given per Pig	Average Body Weight, gm.	Average Pituitary Weight, mg.	Av. R.-T. U. per Pituitary	Av. R.-T. U. per 100 gm. Body Weight	Average % Increase Lactogen per 100 gm. Body Weight	Number of Animals With Open Vagina on Day of Autopsy
10	Controls	296	9.46	3.40	1.15		0
8	5 mg. prgesterone	309	9.54	3.50	1.13		0
5	25 I.U. estrone	316	9.05	4.91	1.55	34.7	4
5	25 I.U. estrone, +5 mg. progesterone	328	9.31	3.41	1.04		2
5	25 I.U. estrone, 5 mg. progesterone	303	9.12	3.50	1.15		1
5	50 I.U. estrone	291	9.36	5.20	1.78	54.7	4
5	50 I.U. estrone, 5 mg. progesterone	330	10.19	4.25	1.28	11.2	2
5	100 I.U. estrone	296	9.44	6.62	2.27	97.3	5
5	100 I.U. estrone, 5 mg. progesterone	298	8.18	4.33	1.45	26.0	3
5	200 I.U. estrone	307	9.78	7.52	2.44	112.1	5
5	200 I.U. estrone, 5 mg. progesterone	329	9.51	7.84	2.38	106.9	5

of animals with vaginal openings were determined in each group of guinea pigs on the day of autopsy.

RESULTS

The data show that the 10 control female guinea pigs contained an average of 3.40 Reece-Turner Units (R-T.U.) or .153 I.U. per pituitary, while the 8 progesterone-treated animals contained an average lactogen content of 3.50 R-T.U. or .157 I.U. per pituitary (table 1). The vaginae of all of the control and progesterone-treated guinea pigs remained closed. Guinea pigs which received 25 I.U. of estrone alone had an average of 4.91 R-T.U. or .22 I.U. of lactogenic hormone per pituitary, which represents an increase of 34.7 per cent. In comparison, two groups of guinea pigs which received a combination of 25 I.U. of estrone plus 5 mg. of progesterone contained no more lactogenic hormone in their pituitaries than did the control guinea pigs. It is of interest that 4 of 5 animals receiving the 25 I.U of estrone alone had open vaginae on the sixth day, whereas in those guinea pigs receiving the combination of 25 I.U. of estrone plus 5 mg. of progesterone only 3 of 10 had open vaginae. Fifty I.U. of estrone

[4] The progesterone was supplied by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J.

alone brought about an average increase of 54.7 per cent in lactogen content and opened the vaginae of 4 of 5 guinea pigs, whereas 50 I.U. of estrone plus 5 mg. of progesterone elicited an average increase in lactogen of only 11.2 per cent and opened the vaginae of only 2 of 5 animals. Administration of 100 I.U. of estrone increased the lactogenic content per pituitary to an average of 97.3 per cent and effected the opening of the vaginae in all 5 guinea pigs treated; this same amount of estrone together with 5 mg. of progesterone kept the lactogen increase down to an average of 26 per cent and reduced the number of guinea pigs with vaginal openings to three. Finally, the injection of 200 I.U. of estrone elicited the greatest increase in lactogenic hormone (112.1 per cent), while the same amount of estrone together with 5 mg. of progesterone failed to reduce the percentage increase in lactogen effected by the estrone alone. The vaginae of all of the animals in both of these groups were opened. This seems to indicate that when the gravimetric ratio of progesterone to estrone is of the order of 250:1, progesterone is no longer able to override the effects of estrone. On the other hand, when the ratio is of the order of 2000:1, progesterone completely overrides the effects of estrone. Reports on the effects which these two sex hormones are capable of exerting on each other generally agree that, in order to override the effects of estrogen, progesterone must be present in much greater quantity.

DISCUSSION

In most animals copious lactation is not initiated during the latter half of pregnancy in spite of the presence of well developed mammary glands. This is believed to be due chiefly to the demonstrated fact that the secretion of lactogenic hormone by the pituitary during pregnancy remains at a low level. This level of lactogen secretion is about the same as in a normal nonpregnant animal. There is no evidence to indicate that the lactogen present is inhibited in any manner from exerting its lactation-inducing effect on the mammary glands. There is simply not enough lactogenic hormone being secreted by the pituitary. Indeed the absence of abundant lactation during pregnancy may be compared to the pseudopregnant state in rabbits. In such animals, at the end of pseudopregnancy, a well developed mammary gland is present also, but usually little or no lactation occurs. Here again, the explanation lies not in a factor which suppresses lactation, but simply in the finding that the lactogen content of the pituitary remains as low as in the normal non-pseudopregnant animal (27). This explains the ease with which such pseudopregnant rabbits can be brought into full lactation following the administration of lactogenic hormone (28–30).

The ability of estrogens (and of testosterone propionate) to stimulate the production of lactogenic hormone by the pituitary has been well established. Yet there is no pronounced increase in the lactogen content of the pituitary during pregnancy in spite of the presence of large quantities of estrogens. There is a possibility that the estrogens of pregnancy may not be present in physiologically active forms, although the only evidence in favor of this view is the finding of inactivated estrogens in pregnancy urine. Inasmuch as the estrogens may undergo considerable metabolic changes in the body, and particularly destruction or conjugation in the liver (31–34), it does not follow that the excretion products found in the urine are representative of the conditions which obtain in the blood stream. Indeed, during pregnancy in women, 50 to 75 per cent of the blood estrogens have been shown to be in the free form (35). A number of considerations make it clear that it is progesterone which is overriding the normal effects of estrogen during pregnancy.

First of all, neither ovulation nor estrum normally occur during pregnancy despite the presence of large quantities of estrogen. That this is due to progesterone is proven

by the fact that *a*), if the corpora lutea are removed during pregnancy in some animals, estrum or ovulation soon follows; *b*), if the life of the corpora lutea is prolonged by hormone treatment or if progesterone is administered beyond the time of normal parturition, parturition itself is delayed, and estrum and ovulation are inhibited (58–60); *c*), normal estrous cycles in such species as the rat have been inhibited experimentally by the administration of progesterone (16, 17). Secondly, in spite of the pronounced stimulating effect of estrogen on uterine motility, the uterus remains very quiescent during pregnancy (61). Ample evidence is available showing that it is progesterone which prevents the action of estrogen on the uterine musculature during pregnancy (24, 36, 61). Third, it has been demonstrated that after ovariectomy in species whose ovaries are essential throughout most of pregnancy, i.e. mice, rats and rabbits, pregnancy can be maintained by the administration of progesterone alone (17, 37–40). While this does not rule out the possible existence of other intrinsic sources of estrogen, it does indicate that progesterone is absolutely essential for the maintenance of pregnancy. In such species as human beings, monkeys and guinea pigs, pregnancy can be maintained following ovariectomy without the administration of progesterone. However, at least in the case of the human female, pregnanediol is continued to be excreted into the urine (41, 42). This indicates that other intrinsic sources of progesterone must be present to maintain pregnancy after ovariectomy in these species. Fourth, in spite of the fact that estrogen can markedly increase the weight of the pituitary, the pituitary weight in the following species during pregnancy has not been shown to be increased, the rat (43–45), the rabbit (2, 46) and the cow (1, 47). This is still another indication that progesterone overrides the effects of estrogen during pregnancy.

It is not implied that all of the effects of estrogen are overridden during pregnancy. Combinations of estrogen and progestin have been shown capable of exerting important synergistic effects on each other as regards the growth of the uterus (24) and mammary glands (26, 62, 63). According to Mixner and Turner, one of the primary functions of estrogen during the occurrence of such a synergism with progestin, particularly as it may affect the growth of the mammary lobule-alveolar system, is to increase the vascularity and permeability of the capillaries of the tissues affected (26). Robson has also suggested an important function for estrogen during pregnancy, namely, that it serves to maintain the life of the corpora lutea (64). This idea is undoubtedly based on the observation that the administration of estrogen may prolong the life of the corpora lutea of rabbits (64–66).

There is good evidence that the removal of the source of progesterone during pregnancy permits estrogen to stimulate an increased elaboration of lactogenic hormone by the pituitary with the consequent onset of lactation. Thus, Drummond-Robinson and Asdell (50) found that if the mammary glands had reached full development in the pregnant goat, ablation of the corpora lutea resulted in immediate and abundant lactation. Selye, Collip and Thomson (51) found that if they removed the intensively luteinized ovaries of rats which had been treated with chorionic gonadotropic hormone and which had developed complete mammary glands, copious milk secretion set in within 36 hours. Allen and Heckel (52) found that if they prolonged the life of the corpora lutea of pseudopregnancy in rabbits by appropriate injection of estrogen, and then removed the ovaries at either the 16th or 25th day and continued injecting estrogen, lactation resulted in every case. Still further proof that progesterone overrides the effects of estrogen on the lactogenic hormone comes from a recent experiment reported by Selye (49). Six adult spayed female rats were injected with 200 micrograms of estradiol and 6 others with the same dosages of estradiol plus 15 mg. of progesterone daily. The mammary glands of the rats which received both hormones were con-

siderably more developed on the 10th day, but milk was found only in the estrogen-treated group. Selye suggested that progresterone directly inhibited the secretion of the lactogenic hormone, while Drummond-Robinson et al. and also Hammond (53) believed that the corpora lutea inhibited lactation. However, it is now clear that progesterone does not inhibit either the secretion of the lactogenic hormone by the pituitary or lactation, but that it only overrides the effects of estrogen on lactogen secretion. Reece and Turner (1) have shown previously, and it has been confirmed in this paper, that the administration of progesterone has no effect on the lactogenic hormone of the pituitary. Anselmino and Hoffmann (54), using a purified preparation of progestin, and Folley and Kon (55), using crystalline progesterone, were unable to suppress established lactation in the rat. Also, Abarbanel reported that large doses of progesterone failed to inhibit lactation in women (67). Finally, the present authors demonstrated that rabbits which were simultaneously lactating and pregnant (and were thus under the influence of the corpora lutea of pregnancy) contained just as much lactogenic hormone in their pituitaries and secreted just as much milk as rabbits which were lactating only (56). It must be emphasized, therefore, that progesterone has no ability to inhibit the secretion of the lactogenic hormone or lactation, but it can, when present in suitable amounts, override certain physiological effects of estrogen.

SUMMARY AND CONCLUSIONS

The following experimental results were obtained with immature female guinea pigs. *a*). Progesterone alone had no effect on the lactogen content of the pituitary. *b*). Estrone alone elicited definite increases in the lactogen content of the pituitary. *c*). Suitable combinations of progesterone and estrone either entirely prevented or reduced the increase in lactogenic hormone which could be obtained with estrone alone. These results are believed to explain why the lactogen content of the pituitary remains as low during pregnancy as in the nonpregnant state. The progesterone-estrogen ratio is believed to be such that progesterone overrides the lactogen-stimulating effects of estrogen. As a result, the amount of lactogenic hormone in the pituitary during pregnancy remains insufficient to initiate an abundant lactation.

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STUDIES CONCERNING THE MECHANISM CONTROLLING THE INITIATION OF LACTATION AT PARTURITION[1,2]

III. CAN ESTROGEN ACCOUNT FOR THE PRECIPITOUS INCREASE IN THE LACTOGEN CONTENT OF THE PITUITARY FOLLOWING PARTURITION?

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THE FOLLOWING FACTS have been established concerning the relation of the lactogenic hormone to the initiation of lactation. *a*). Estrogen can increase the secretion of lactogenic hormone by the pituitary and initiate lactation. *b*). During pregnancy there is little if any increase in lactogenic hormone despite the presence of large amounts of estrogen. This is attributed to the predominant quantity of progesterone which over-rides certain effects of estrogen during pregnancy. Evidence will now be submitted showing that the diminution or cessation of progesterone secretion just prior to parturition permits estrogen to assume the ascendency and quickly stimulate an increased elaboration of lactogenic hormone, which in turn brings about the onset of lactation.

There is considerable evidence in favor of the view that estrogen plays a major rôle in the initiation of labor, and it is not unexpected that this event should be closely associated with the initiation of milk secretion. Allan and Dodds (1), Bourne and Burn (2), and Jeffcoate (3) have suggested the theory that pregnancy continues while progesterone is in the ascendency, but that if estrogen becomes dominant the pregnancy is terminated. In favor of this view are the findings that the administration of estrogens can terminate pregnancy in the mouse (4), rat (5), rabbit (6), and cow (7), while the administration of progesterone or the production of new corpora lutea results in the prolongation of pregnancy (8–13). It is true that estrogens administered to women do not seem to be very effective in terminating pregnancy, but this may be caused by rapid inactivation of the estrogens as suggested by the excretion of large amounts of inactivated estrogens in the urine during pregnancy. It is rather paradoxical that the life of the corpora lutea can be prolonged by administering certain dosages of estrogens (13–16). However, Heckel and Allen (13) found that dosages of estrogen which were sufficient to prolong the life of the corpora lutea and delay parturition in pregnant rabbits always caused death of the fetuses. These authors concluded that the corporea lutea in the rabbit must become nonfunctional before parturition can occur. This conclusion is corroborated by the observation that *a*), parturition in women is preceded by a fall in pregnanediol excretion in the urine (17–20) while in sows the progestin content of the corpora lutea drops to nothing some 10 days before parturition (21), and *b*), cases of habitual abortion are often preceded by a similar decline in pregnanediol excretion (4). Recurrent abortion has been successfully treated with progesterone therapy (22–24).

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The rôle of estrogen in the initiation of parturition is excellently illustrated in the case of the rabbit. Rhythmical contractions of the uterus occur only when the doe is in heat; ovariectomy results in loss of uterine motility within a few days, while estrogen administration can bring a restitution of uterine motility within 24 hours (25). Quoting from Reynolds (26): "It is not possible to account for the slight but definite increase in uterine activity and reactivity towards the end of pregnancy in the rabbit at a time when the corpus luteum is somewhat functional except on the grounds that estrin is activating the musculature of the uterus. Another line of evidence lies in the fact that towards the end of pregnancy a unilaterally sterile horn shows a pattern of motility which is exactly like that seen in the nongravid uterus during estrus so proving beyond doubt that it is estrin which is activating the uterus at this time."

Additional proof of high estrogen activity at parturition is afforded by the following lines of evidence *a*). Rabbits come into heat immediately after parturition, and such does can be bred without difficulty during the first few days post partum (27). *b*). Ovulation occurs in the rat and mouse immediately following parturition (28, 29). *c*). The pituitary in some animals increases in size soon after parturition, and this can be at least partially attributed to estrogen. *d*). The lactogen content of the pituitary is increased by several hundred per cent within a few days following parturition, and a major portion of this increase occurs whether or not suckling takes place (30, 31).

There is some belief that the posterior pituitary plays a part in the initiation of labor, since upon experimental administration it can undeniably exert an oxytocic effect on the uterus. This idea may be questioned, however, in view of the demonstration that parturition can proceed normally in cats (32), rats (33–35) and mice (36) after the removal of the posterior lobe or the entire pituitary during pregnancy. In a recent article (37), Petersen suggested that the posterior pituitary may be the factor responsible for the increased elaboration of lactogen following parturition, and also for the decrease in the lactogen content of the pituitary observed after suckling in the lactating rat, guinea pig and rabbit. The present authors have found, to the contrary, that *a*), the administration of large doses of pituitrin failed to increase the lactogen content of the pituitary of 8 immature male guinea pigs; *b*), large amounts of pituitrin failed to decrease the lactogen content of the pituitary of 4 immature male guinea pigs whose lactogen content had previously been raised to the lactation level with estrone; and *c*), a single lactating rabbit was injected intravenously with pituitrin over a period of 15 hours, and failed to show any alteration in the lactogen content of the pituitary (31). These results would indicate that the posterior pituitary is not responsible for the great increase in the lactogen content of the pituitary following parturition, or for the decrease in the lactogen content of the pituitary observed after nursing.

It is believed that estrogen (and also testosterone propionate), which has thus far been shown to be the only hormone capable of eliciting an increase in the lactogen content of the pituitary and blood stream, and initiate lactation, is the factor primarily responsible for the quick rise in the lactogen content of the pituitary of rats, guinea pigs and rabbits 2 to 5 days post partum. It was desirable to determine, therefore; *a*) whether or not the experimental administration of estrogen during a correspondingly short period of time would also elicit a notable increase in the lactogen content of the pituitary; and *b*), whether or not hysterectomy on the twentieth day of pregnancy in the rabbit, leaving the ovaries intact, would bring an increase in the lactogen content of the pituitary. Previously it had been shown that removal of the ovaries on the twentieth day of pregnancy in the rabbit caused no increase in the lactogen content of the pituitary 2 to 5 days later (38) It now seems likely that this failure to secure a rise in lactogenic hormone was due to the deprivation of the principal source of estrogen in the rabbit, since it has not been possible to demonstrate the presence of estrogen in the rabbit placenta. Greep (15) found that a lipid extract of 240 gm. of rabbit placentae taken from does 15 to 25 days pregnant was unable to induce an estrus smear in a spayed adult female rat. Since hysterectomy after the fifteenth

day of pregnancy in the rabbit has been shown to induce quick degeneration of the corpora lutea (15), it logically follows that the estrogen secreted by the ovaries should be free to stimulate an increase in the lactogen content of the pitutitary.

PROCEDURE AND RESULTS

Estrogen Experiment with Guinea Pigs

Forty male guinea pigs were divided into eight groups of 5 each, and a total of 0.3 mg. of estrone (3000 I.U.) was injected subcutaneously into each animal during increasing periods of time. At the end of the period of injection each group of animals was killed, and the pituitaries were removed for assay. The periods of injection ranged from 1 to 30 days.

Results. These data (table 1) show that when 0.3 mg. of estrone was given in a single dose, or divided into 4 equal doses over a 2-day period, the average lactogen

TABLE 1. EFFECT OF LENGTH OF ESTROGEN TREATMENT ON LACTOGENIC CONTENT OF THE PITUITARY
All animals received a total of 0.3 mg. of estrone or 3000 I.U.

Number of Guinea Pigs	Length of Treatment	Average Body Weight	Average Pituitary Weight	Average Reece-Turner Units,[1] per			Average % Increase in R.-T. Units per 100 gm. Body Weight
				Pituitary	mg. Pituitary Tissue	100 gm. Body Weight	
	days	*gm.*	*mg.*				
11	Controls	337	9.86	3.06	.31	.90	
5	One injection, killed 48 hours later	321	11.36	7.00	.61	2.18	142
5	Twice daily for 2 days, killed 3rd day	328	12.45	9.00	.72	2.74	204
5	5	365	13.54	15.50	1.14	4.24	371
5	10	374	14.85	16.00	1.07	4.54	404
5	15	310	12.65	14.25	1.12	4.59	410
5	15 days, killed 10 days later	421	15.41	13.85	.89	3.29	265
5	20	423	16.04	15.75	.92	3.72	313
5	30	418	15.35	17.75	1.15	4.24	371

[1] Reece-Turner lactogen units. One R-T. U.= .045 international units (MEITES, J., A. J. BERGMAN AND C. W. TURNER: *Endocrinology* 28: 707, 1941.

content per pituitary rose from 3.06 Reece-Turner (30) units (.138 I.U.) to 7.00 (.315 I.U.) and 9.00 R.T. U. (.405 I.U.) respectively. On the basis of lactogen units per 100 gm. of body weight, this represents an average increase of 142 and 204 per cent respectively. A 5-day period of injection increased the average lactogen content per pituitary to 15.50 R.T. U. (.698 I.U.), which on a body weight basis represents a 371 per cent increase over the control animals. Those guinea pigs which were injected for 10 to 30 day-periods showed approximately the same rise in pituitary lactogen as the 5-day group. This seems to indicate that the maximum stimulation with estrogen on the lactogenic potency of the pituitary can be secured as well in a 5-day period as during a longer time interval. This is the reason 5-day-injection periods were used in studying the estrogen effects on pituitary lactogen in the previous two publications (39, 40). It is interesting that the average lactogen content in the pituitaries of the group which was injected for 15 days and killed 10 days later remained high, suggesting that the effects of estrogen can be carried over considerably beyond the period of injection.

These data indicate that estrogen is capable of exerting a remarkably quick stimu-

lus on the lactogen production of the pituitary. It is believed that this mechanism accounts for the rapid increase in the lactogen content of the pituitary observed in the rat and guinea pig during estrum, and in the rat, guinea pig and rabbit two to five days post partum.

Hysterectomy Experiment with Pregnant Rabbits

Four New Zealand White does were hysterectomized (removing the uterus, placentae and fetuses) on the twentieth day of pregnancy, care being taken to leave the ovaries and their blood supply intact. The rabbits were killed 5 days after the operation, the pituitaries were removed, and each pituitary was individually assayed for lactogen by the Reece-Turner method.

TABLE 2. LACTOGEN CONTENT OF ANTERIOR PITUITARY OF RABBITS HYSTERECTOMIZED ON 20TH DAY OF PREGNANCY AND KILLED 5 DAYS LATER

Rabbit No.	Body Weight	Pituitary Weight	Reece-Turner Units, per			Per Cent Increase of Lactogen per 100 gm. Body Weight
			Pituitary	mg. Pituitary tissue	100 gm. Body weight	
	gm.	mg.				
1	3570	41.95	16.80	.40	.47	79
2	3263	32.61	16.00	.49	.49	80
3	3006	34.00	14.75	.43	.49	80
4	3101	24.50	16.50	.67	.53	95
Average	3235	33.26	16.01	.49	.49	83

Two previous studies of the lactogen content of the rabbit pituitary on the twentieth day of pregnancy showed that it contained 10.63 and 9.75 R.T. units, or approximately 10 R.T. units (38). The does which were ovariectomized on the twentieth day of pregnancy, and killed 2 and 5 days later, averaged 10.70 and 10.75 R.T. units of lactogen per pituitary, indicating that no increase in lactogen was obtained as a result of ovariectomy.

Results. It will be seen (table 2) that in the case of the hysterectomized rabbits, their average lactogen content per pituitary rose to an average of 16.01 R.T. units 5 days after the operation. Although this increase (83%) is relatively small compare to that which occurs post partum (400% or more), and practically no lactation was initiated, it is believed to be significant since it indicates that the corpora lutea must regress before estrogen can stimulate lactogenic production. It is of interest that the lactogen content of the pituitary of pregnant rabbits also rises slightly (to 14.10 R.T. units) on the twenty-eighth day of pregnancy, reflecting a decline in progesterone activity and an increase in estrogen activity. That the corpora lutea regress in the rabbit beginning about the twenty-seventh day of pregnancy seems to be indicated from the findings of Knaus (41) and Reynolds (26) that the uterine sensitivity to pituitrin and other oxytocic agents remains low during most of pregnancy but increases sharply beginning on the twenty-eighth day of gestation. It seems very probable that hysterectomy and removal of the conceptus in the rabbit at a stage later than the twentieth day of pregnancy would have resulted in a greater increase in lactogenic hormone.

DISCUSSION

From the study of the lactogen content of the pituitary it would appear reasonable to believe that it serves as a good measure of its secretion rate under various

influences. The significant discovery was made that the lactogen content of the pituitary was just as low during pregnancy as in the nonpregnant state, whereas immediately following parturition there was a tremendous increase in the lactogenic hormone. This made it clear, then, that *a*), lactation was intimately associated with a considerable increase in lactogen production; and *b*) some activating factor at parturition was responsible for the increassed output of lactogen by the pituitary. Up to the present time in studying the influence of other hormones on the lactogen content of the pituitary, only the estrogens (and testosterone propionate) were found capable of stimulating an increased elaboration of lactogen by the pituitary. Pituitrin (31), progesterone (30, 40), adrenal cortical extracts and tissue (31), and desoxycorticosterone acetate (42) have been shown to have no effect on the lactogen content of the pituitary. Injections of large amounts of thyroxine into rats may have resulted in a slight reduction in the lactogen content of the pituitary (30), although thyroxine failed to cause any change in the excretion of lactogen into the urine of lactating goats (43). Thus, it seemed most probable that estrogen was the inciting factor at parturition which increased lactogenic secretion and initiated lactation. It was difficult to understand, however, why lactogen production continued at such a low rate during pregnancy when large amounts of estrogen were known to be present. The appreciation of the predominant action of progesterone over estrogen during pregnancy provided the obvious answer, namely that the exciting effect of estrogen on lactogenic secretion was being over-ridden by progesterone. The reduction and disappearance of progesterone just prior to and during parturition, then, made it possible for estrogen to initiate lactation.

The relatively simple explanation of the mechanism initiating lactation at parturition which has just been outlined does not, of course, provide the entire answer. An important prerequisite for lactation is the presence of a well developed mammary gland. Also, hormones other than lactogen undoubtedly exert important supplementary effects on lactation, especially on the precursors of milk.

If a lactating animal is hypophysectomized, lactation stops almost immediately. The administration of lactogen alone to such animals will not re-initiate lactation, but lactogen plus cortin or adrenotropin will reinitiate lactation (44–46). This indicates that the adrenals are an important factor in the initiation and maintenance of lactation. Adrenalectomy studies seem to indicate that considerably more cortin is necessary to maintain lactating than non-lactating rats (47). If only enough cortin is given to keep the animals alive they will not lactate. In the case of the pseudopregnant rat, cortin as well as lactogen is needed to initiate lactation (48). Brownell et al. (49, 50) claimed that there was a specific factor in the adrenals, 'cortilactin,' which was essential for lactation. Not only has this failed to be substantiated by other workers, but it has been demonstrated that some lactation can be maintained in adrenalectomized animals by the administration of salt alone (47, 51). It is possible that there may be an increase in cortin as well as in lactogen secretion at parturition, and estrogen may be responsible for both effects. Several investigators have reported an increase in the size of the adrenals of rats following estrogen administration (52–55). The available data indicate that the adrenals exert their favorable effects on lactation chiefly through their influence on salt, water and carbohydrate metabolism rather than through any direct effect on the lactogenic hormone or the mammary glands.

The thyroid is not necessary for the initiation of lactation, but it can exert a definite supplementary influence on lactation. Removal of the thyroid during pregnancy does not prevent the onset of lactation (56, 57), but thyroidectomy during lactation can reduce the amount of milk secreted (58). On the other hand, the admin-

istration of thyroid tissue or thyroxine during lactation produces a considerable increase in milk yield (58–61). It is also of interest that a 40 per cent increase in the thyrotropin content of the rat pituitary has been reported following parturition (62).

It is probable that other endocrines such as the parathyroids and pancreas, as well as the metabolic hormones of the pituitary, also exert important supplementary influences on lactation. The reports on the augmentation of established lactation make it clear that whole pituitary extracts are more effective than lactogen alone. However, hormones other than lactogen cannot be considered lactogenic hormones since they alone will not initiate lactation. In pseudopregnant rabbits, less lactogen is required to initiate milk secretion when other pituitary hormones are also given, but large amounts of these other pituitary hormones with little lactogen will not initiate lactation (63).

After lactation is initiated at parturition by the mechanism described, another mechanism plays the principal rôle in maintaining lactation. The stimulus of nursing is believed to be responsible for the maintenance of lactation (64–66), and this has been shown to be through a direct action on the secretion of lactogenic hormone by the pituitary (67–69). That estrogen is not necessary to maintain a high lactogen secretion post partum is proven by the demonstration that lactation can proceed normally following ovariectomy (70). The presence of the nursing stimulus explains why lactation and pregnancy can proceed together at the same time.

Considerable evidence is available which indicates that the act of suckling alone may initiate lactation under certain conditions. Thus, Turner (70) reported the initiation of lactation in virgin and nonpregnant cows following regular manipulation of the teats and removal of the secretion present. Selye et al. (71) reported the growth of the mammary glands and the initiation of lactation in normal rats and mice following constant stimulation of the nipples by actively suckling litters. The anthropologist, Mead (72), observed that it was a regular custom among women of the Mundugumor tribe of New Guinea to suckle adopted infants. She says: "Even women who have never borne children are able in a few weeks, by placing the child constantly at the breast and by drinking plenty of cocoanut-milk, to produce enough or nearly enough milk to rear the child, which is suckled by other women during the first few weeks after adoption." It must be emphasized, however, that the nursing stimulus is not essential for the initiation of lactation following parturition. It has been demonstrated in rats (30), guinea pigs and rabbits (31) that the lactogen content of the pituitary rises and milk secretion begins after parturition even in the absence of nursing. Furthermore, it is well known that spontaneous lactation sometimes occurs in normal animals (38), presumably because of hyper-estrogen or hyper-testosterone stimulation of the pituitary.

It is possible to postulate, on the basis of the theory of the initiation of lactation which has been presented, some of the possible causes for the rather frequent cases of hypogalactia observed in women and animals following parturition. Hypogalactia may be due to *a*), insufficient development of the mammary apparatus; *b*), refractoriness of the pituitary to estrogen stimulation at parturition; or *c*), insufficient estrogen at parturition to augment the lactogen production by the pituitary to the extent needed for copious lactation. Lactogen and whole pituitary extracts have been used to correct such deficiencies and increase milk production with varying degrees of success. Another possible therapeutic measure would be the administration of moderate doses of estrogen to stimulate increased lactogen production by the animal's own pituitary. That such therapy may be successful in overcoming inadequate lactation is suggested by the ability of diethylstilbestrol to initiate milk secretion in goats and cows (73–75).

Walker and Stanley (75) found that they were able to secure an increase in milk production in cows during the administration of moderate amounts of diethylstilbestrol, but decreases in milk yield were obtained when large doses of the hormone were given. The greater effectiveness of small over large doses of estrogen in augmenting lactation is probable and seems indicated from *a*), the demonstration that smaller quantities of estrogen can increase the lactogen content of the pituitary to a greater degree than larger quantities, and *b*), the possibility that large quantities of estrogen may so reduce the secretion by the pituitary of hormones other than lactogen, that a decrease rather than an increase in lactation would be obtained.

SUMMARY AND CONCLUSIONS

The effect of length of estrogen treatment on the lactogen content of the pituitary was determined in 40 male guinea pigs, divided into eight equal groups. All guinea pigs received the same total amount of estrone (0.3 mg.) over periods of time varying from 1 to 30 days. A 142 and 204 per cent increase in lactogenic potency of the pituitary was obtained in the groups injected for 1 and 2 days respectively, whereas a 371 per cent increase was secured in the animals injected for 5 days. The animals injected for 10 to 30 days did not show a significantly greater increase than was observed in the 5-day group. These results indicate that estrogen can account for the rapid increase found in the lactogen content of the pituitary of rats, guinea pigs and rabbits 2 to 5 days post partum.

Four New Zealand White rabbits were hysterectomized on the twentieth day of pregnancy and were killed 5 days later. Individual assays of the pituitaries revealed than an average increase of 83 per cent of lactogenic hormone had been obtained, indicating that the predominant influence of progesterone during pregnancy must be removed before estrogen can effect an increase in the lactogen content of the pituitary.

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CONDITIONS MODIFYING THE EFFECTIVENESS OF TESTOSTERONE, TESTOSTERONE PROPIONATE, AND METHYL TESTOSTERONE

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AS A RULE, the potencies of the various physiologically active steroids have been compared on the basis of their actual weights. Either effects obtained by identical milligram amounts are graded, or those milligram or microgram doses which bring about equal effects are considered equi-effective. There is no objection to such methods as long as the substances compared have at least approximately the same molecular weights. The differences in the molecules of testosterone and androsterone or estradiol and estrone are so small, that they become insignificant in relation to other factors influencing the results in animal experimentation. Thus in many instances, comparisons based on equal molecular amounts will not differ essentially from those based on equal milligram amounts. If, however, substances with weights deviating 15 per cent and more are being compared, it would seem worthwhile to take such differences into account, particularly when the part of the molecule causing the increase in weight is known to be itself inactive, as is the case with various esters of active sterols.

Some of the present authors (1) have demonstrated that testosterone, percutaneously applied, in a certain ointment was significantly more effective than equal milligram amounts of testosterone propionate, although when equal molecular amounts were used, no difference in the effects of the two substances was found. In another paper(2), the effectiveness of testosterone and testosterone propionate in different vehicles by the percutaneous and subcutaneous routes was studied. The data for this study were obtained, however, by using equal milligram amounts, and the conclusions thereby lose much of their value. A comparative study of the efficacy of equal molecular amounts of testosterone, testosterone propionate, and methyl testosterone has been presented recently (3). Only the results obtained by the percutaneous route were reported, and it was shown that both the actual and the relative potencies of these substances, when tested in 3 different ointments, varied according to the particular ointment vehicle in which they were applied.

The present study represents in part a continuation of the previous study on the effectiveness of these three androgens by the percutaneous route. In addition, however, data were collected on the effects of these three compounds administered subcutaneously in oil and subcutaneously in pellet form. Data obtained from 458 treated, castrated rats (278 of which were reported upon in the previous study) are utilized in

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forming conclusions concerning the relative and actual potencies of these androgens in different vehicles and by different routes of administration.

PROCEDURE

Male rats weighing 150 ± 10 gm. were castrated and divided into groups having 19 to 22 animals in each. The groups were treated daily for 21 days, either subcutaneously or percutaneously, or by implantation of pellets of testosterone, testosterone propionate, and methyl testosterone. Treatment was begun the day after castration, and the animals were killed with ether on the day following the last treatment. Ventral prostates and seminal vesicles (without coagulating glands) were removed by careful dissection and immediately weighed.

For the subcutaneous administration, equal molecular amounts of each androgen (0.2 mg. of testosterone, 0.239 mg. of testosterone propionate, and 0.21 mg. of methyl testosterone) in 0.1 cc. of sesame oil were used. The same quantities were administered percutaneously in 0.05 cc. 95 per cent alcohol and in 0.11 cc. of ointment. The components of the ointments have been given previously (3). In the same way as reported in a previous communication (3), the quantity of alcohol and ointment, measured from a tuberculin syringe, was applied directly onto a clipped area of the back of the animal. A fine-gauge needle was used for the alcoholic solution to localize the application. The ointments were rubbed evenly over the clipped area with the barrel of the syringe until no excess of the ointment was left on the surface.

The pellets of each of the androgens were made by compression of the pure crystalline substances in a mold[1] resulting in small thin cylinders, the weights of which, of course, varied to some extent. In each instance, however, they were accurately weighed and a fairly uniform group were selected for implantation. The desired initial weight of the testosterone pellets was calculated (6.0 mg.) to provide a probable daily absorption comparable to that furnished by the other methods of treatment. The initial weights of the testosterone propionate and methyl testosterone pellets were adjusted so that equal molecular amounts of the hormones could be implanted. Data concerning the pellet weights and absorption of the hormones therefrom are given in table 4. The pellets were removed at autopsy, cleaned, and dried in an oven at 50° C. for a few days before weighing.

RESULTS

The mean body weights and the standard deviations from the mean were calculated for each experimental group. They varied from 178 ± 2.34 to 192.5 ± 2.87 gm. In no instance was the difference between any two groups significant. To simplify the tables the body weights therefore were not included.

Relative effectiveness of the androgens administered percutaneously in alcohol. When applied percutaneously in alcohol, the non-esterified testosterone produced the largest mean prostate and seminal vesicle weights, as seen in table 1. In no instance, however, were the differences with regard to the effect on the prostate significant. Testosterone, however, produced seminal vesicles significantly heavier than did testosterone propionate, while the difference in effect between testosterone and methyl testosterone was again not significant.

There are no comparable data based on equal molecular amounts available in the literature. Miescher and Gasche (4), however, using equal milligram amounts (and thus relatively less testosterone propionate) have concluded that there is no significant

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[1] The authors wish to thank Dr. T. R. Forbes for his kindness in preparing the pellets according to the method described in his publication (*Endocrinology* 29: 70. 1941).

TABLE 1. EFFECTS OF PERCUTANEOUS ANDROGENS IN ALCOHOL

Substance	Number of Animals	Prostate			Seminal Vesicle		
		Mean and σ mg.	Critical ratio		Mean and σ mg.	Critical ratio	
			T.P.	T.		T.P.	T.
Testosterone Proprionate	19	247±14.8		2.43	572±49.2		**3.63**
Testosterone	20	297±14.4			782±30.4		
Methyl Testosterone	22	262±12.96	0.76	1.81	721±33.9	2.49	1.34

Standard deviation and critical ratio were calculated as set forth in previous papers, $N(N-1)$ being used for groups of 20 or more animals, $N(N-2)$ for smaller groups. Significant critical ratios in bold face type. (2.86 or more in groups of this size).

difference in the percutaneous effect of testosterone and testosterone propionate in alcohol. This is directly at variance with the findings reported here. Part of their data was obtained by administering 1 mg. per day of each of the two substances in alcohol. This is an exceptionally large dose, particularly in this vehicle, and it would be difficult to show quantitative differences between the two androgens with this dose, since the animals would respond maximally to both. In support of this criticism, we refer to a previous demonstration (2), wherein a significant difference was observed between testosterone and testosterone propionate percutaneously administered in alcohol, when the dose was 0.2 mg. but not when the daily dose was 0.6 mg. Another part of Miescher and Gasche's data which is at variance with our findings was obtained with doses of 0.2 mg. daily. The foregoing explanation of course is not applicable to this part of their findings.

Relative potency of androgens administered percutaneously in alcohol and in ointments. For comparative purposes, part of the data of a previous paper (3) reporting the effect of the three androgens in three types of ointments, have been included in table 2. The figures for the weights of prostate and seminal vesicle obtained with the three androgens dissolved in alcohol are compared with the corresponding figures resulting from the experiments with the androgens in ointment form. Because the efficacy of the compounds varies with the type of ointment used, we have selected for this table the ointment in which the specific hormone had the greatest effect. As may be noted in the table, compared with the optimal ointment, each androgen is significantly more effective when administered in alcohol. That testosterone and tetosterone propionate,

TABLE 2. COMPARISON OF PERCUTANEOUS ANDROGENS IN ALCOHOL AND IN MOST EFFECTIVE OINTMENT

Substance	Vehicle	Prostate		Seminal Vesicle	
		Mean and σ mg.	Critical ratio	Mean and σ mg.	Critical ratio
Testosterone Propionate	Alcohol	247±14.8	**4.74**	572±49.2	**5.75**
	Protegin	172± 5.6		283±10.3	
Testosterone	Alcohol	297±14.4	**6.30**	782±30.4	**8.36**
	Vodol	195± 7.5		422±30.5	
Methyl Testosterone	Alcohol	262±13.0	1.52	721±33.9	**4.33**
	Vodol	239± 7.8		568±31.2	

TABLE 3. EFFECTS OF ANDROGENS ADMINISTERED SUBCUTANEOUSLY IN OIL

Substance	Number of Animals	Prostate			Seminal Vesicle		
		Mean and σ mg.	Critical ratio		Mean and σ mg.	Critical ratio	
			T.P.	T.		T.P.	T.
Testosterone Propionate	22	332±17.4			872±32.9		
Testosterone	20	157±10.3	8.65		197±19.4	17.68	
Methyl Testosterone	21	171±11.8	7.65	6.89	222±27.0	15.82	0.75

by the percutaneous route, are most effective in alcohol is in agreement with the findings previously reported (2). Similar observations for the same substances have been presented by Miescher and Gasche (4), and for testosterone by Zondek and Sulman (5). It seems worth noting that methyl testosterone in vodol, which was found to be the most active androgen in ointment form, is still significantly less effective than testosterone and methyl testosterone administered in alcoholic solution. Testosterone propionate in alcohol, however, showed no greater potency than did methyl testosterone in vodol.

Relative effectiveness of the androgens administered subcutaneously in oil. The effects of the three androgens on the prostate and seminal vesicle when administered subcutaneously in equal molecular amounts in oil are presented in table 3. No special consideration is needed, because it is well established that testosterone propionate is much more effective than testosterone or methyl testosterone. Because here for the first time the three subcutaneously administered androgens are compared, on the basis of their molecular weights, it is, however, worth noting that testosterone propionate produced prostates twice as large and seminal vesicles four times as large as those produced by testosterone and methyl testosterone. The mean prostate and seminal vesicle weights were slightly higher after administration of methyl testosterone than after testosterone, but the differences were not significant in either case.

Data on the comparative potency of methyl testosterone and testosterone in the subcutaneously-treated rat have been given by Deanesly and Parkes (6), Miescher, Wettstein and Tschopp (7), and by Emmens and Parkes (8). Methyl testosterone was reported in each publication to be slightly more effective when compared milligram for milligram. These data, although not subjected to statistical analysis, were obtained from multiple groups of animals at different dose levels, and consistent differences between groups were observed. It, therefore, seems likely that the differences we found in our experiments with testosterone and methyl testosterone, while not

TABLE 4. EFFECTS OF SUBCUTANEOUS ANDROGEN PELLETS

Substance	Number of Animals	Initial Pellet Weight mg.	Average Daily Absorption mg.	Prostate			Seminal Vesicle		
				Mean and σ mg.	Critical ratio		Mean and σ mg.	Critical ratio	
					T.P.	T.		T.P.	T.
Testosterone Propionate	19	7.12	0.13	397±17.1			1149±43.3		
Testosterone	22	6.03	0.18	423±13.8	1.14		1184±34.4	0.63	
Methyl Testosterone	21	6.30	0.18	371±20.1	0.99	2.13	1082±43.1	1.0	1.85

statistically significant, are due to an actually greater subcutaneous effectiveness of methyl testosterone.

Relative effectiveness of the androgens in pellet form. In table 4 are shown the data obtained with the three androgens when given in equal molecular amounts in pellet form. This does not mean, however, that 'equal molecular amounts' have been absorbed. This term refers only to the original weights of the pellets implanted. The average amounts absorbed daily are included in the table. The amounts absorbed daily from the pellets of testosterone and methyl testosterone were equal, but lower in the case of testosterone propionate.

While some differences in the mean prostate and seminal vesicle weights were

TABLE 5. EFFECTS OF ANDROGENS ADMINISTERED SUBCUTANEOUSLY IN OIL, PERCUTANEOUSLY IN ALCHOL AND AS SUBCUTANEOUS PELLETS

Substance	Administration	Prostate			Seminal Vesicle		
		Mean and σ mg.	Critical ratio		Mean and σ mg.	Critical ratio	
			Subcut.	Pellet		Subcut.	Pellet
Testosterone Propionate	Subcutaneously	332±17.4		2.67	872±32.9		5.09
	Pellets	397±17.1			1149±43.3		
	Percutaneously	247±14.8	3.72	6.64	572±49.2	5.07	8.81
Testosterone	Subcutaneously	157±10.3		15.46	197±19.4		25.00
	Pellets	423±13.8			1184±34.4		
	Percutaneously	297±14.4	7.93	6.33	782±30.4	16.24	8.76
Methyl Testosterone	Subcutaneously	171±11.8		4.29	222±27.0		16.91
	Pellets	371±20.1			1082±43.1		
	Percutaneously	262±12.96	5.19	4.56	721±33.9	11.51	6.58

found with the three different types of pellets, none of them was significant. These data, however, afford no conclusions as to the relative effectiveness of equal molecular amounts of these androgens in pellet form. The organ weights in each group were so high that it seems likely that the amount of each hormone implanted evoked a maximal, or at least nearly maximal response. To establish possible differences in the effects of the three androgens, pellets should be used with an absorption rate lower than that which we obtained in our experiments.

Biskind and Meyer (9) have studied the relative potency of these three androgens in pellet form. Using adult castrates, they attempted to compare the effectiveness of equal absorbed amounts of the three hormones. However, the absorbed quantities in their experiment were similar to those of ours with testosterone and methyl testosterone, and the resultant organ weights were of such magnitude that comparisons become impossible because the weights probably represent a maximal or nearly maximal response. It is, therefore, not surprising that these authors found no 'marked difference' among the three androgens.

The same authors using younger animals also compared the effects of pellets. The weight of the pellets and the quantity absorbed were not equal, either on the basis of molecular weight or actual weight.

To our knowledge no adequate study of the relative effectiveness of these three androgens in pellet form has been published.

Effectiveness of each androgen as influenced by the route of administration. In table 5, we have compiled the weights of the accessory organs after administration of each

androgen by the various routes—subcutaneously in oil, in pellet form, and percutaneously in alcohol. The fact that in each instance the pellet form is the most effective is obvious, and needs no further discussion. As expected also, testosterone propionate is more effective subcutaneously in oil than percutaneously in alcohol. The non-esterified testosterone and methyl testosterone, however, proved to be more potent dissolved in alcohol and given by inunction than subcutaneously in oil.

Relative potency of the three androgens. It is our opinion that many misconceptions concerning the relative potency of the different androgens have arisen, and the chief reasons are, first, that the comparisons have been made on a milligram basis instead of on a molecular weight basis, and second, that the conditions of the experiments have not been adequately considered. The importance of the conditions under which the hormones are administered is shown in the following compilation. It is apparent that the relative potencies of the three androgens change as the conditions of administration are changed. The relative and the actual efficacy of androgens are modified not only by the route of administration, but, in the case of the percutaneous route, also by the particular vehicle employed. This opens the question of whether the relative potencies by the subcutaneous route may not also be altered by the use of different vehicles.

Route of Administration	Vehicle	Order of Effectiveness (Seminal Vesicle)
Percutaneous[1]	Ointment (H–O)	MT>>TP>T
Percutaneous[1]	Ointment (Protegin)	MT>T>>TP
Percutaneous[1]	Ointment (Vodol)	MT>>T>>TP
Percutaneous[2]	Alcohol	T>MT>TP
Subcutaneous[2]	Sesame oil	TP>>MT>T
Subcutaneous[2]	Pellets	T>MT>TP
Oral[2]	50% Alcohol	MT>T>TP

T, testosterone; TP, testosterone propionate; MT, methyl testosterone; >>=significantly larger seminal vesicle weight; >=larger seminal vesicle weight.

[1] Data from previous publication (3).

[2] Data from present paper.

[3] Data from Miescher and Tschopp (10).

SUMMARY AND CONCLUSIONS

Equal molecular amounts of testosterone, testosterone propionate, and methyl testosterone have been administered to castrated male rats by the subcutaneous and percutaneous routes, and in the form of subcutaneously implanted pellets. Each of the three androgens was administered percutaneously in four different vehicles, three types of ointments and alcohol. The effect on prostate and seminal vesicle weights after 21 days of daily treatment was used as the criterion of effectiveness. The findings were as follows.

1. Each androgen was most effective when administered in pellet form.

2. Testosterone and methyl testosterone were both more effective when given percutaneously in alcohol than subcutaneously in oil, while testosterone propionate was more effective subcutaneously than percutaneously.

3. Each androgen was percutaneously more effective in alcohol than in ointment.

4. Applied percutaneously in alcohol, testosterone and methyl testosterone were not significantly different, but testosterone was significantly more effective than testosterone propionate.

5. Subcutaneously in oil, testosterone propionate was the most effective, and

methyl testosterone slightly, but not significantly more effective than testosterone.

6. In the amounts implanted there were no significant differences in the effects of pellets of the three androgens.

From these and other quoted data, it is concluded that the potency of each of the androgens and also their relative potencies are markedly modified by the route of administration and, percutaneously, by the vehicle used.

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EFFECTS OF ESTROGENS UPON THE YOUNG OF INJECTED LACTATING RATS[1]

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MANY INVESTIGATORS have studied the effects of estrogens injected into lactating females. Most of these were interested in lactation *per se* and paid little attention to the effects on the young other than on their rate of growth. It has been shown, however, that injection of large doses of estrogens into lactating rats over the first 4 or 5 days after parturition results in a permanent modification of the external genitalia of the female young (1–4). The present report is concerned with some more extensive effects on the young of estrogen injected into lactating rats.

MATERIALS AND METHODS

The litters of each of 15 rats were reduced to 6 (3 males and 3 females) immediately after parturition. The weights of each mother and litter were recorded daily throughout the experiment.

In order to observe the effects of estrogens in different amounts, 3 rats were given daily subcutaneous injections of .025, .05 and .1 mg. of theelin and 3 others were similarly injected with diethylstilbestrol beginning on the day of parturition. In addition, 1 animal was injected with .5 mg. of theelin daily. The young of these rats were autopsied on the 14th day.

In order to determine the earliest time at which estrogens, administered to the mother, would affect the young, 2 rats were injected daily with .1 mg. of theelin and 2 others with .1 mg. of diethylstilbestrol. Male and female offspring were autopsied on *days* 2, 4, 6, 8, 10, and 12. Control young were also obtained for these days.

All urogenital organs were fixed in Bouin's solution. Sections of the ovaries, Fallopian tubes, uteri, vaginae, bladders and urethrae of the female young were prepared for study. In the males, the testes, epididymides, prostate glands, seminal vesicles, Cowper's glands, bladders and urethrae were sectioned.

OBSERVATIONS

Weight of Young

There is a decided deleterious effect upon the growth of the suckling young of estrogen-injected mothers. The animals grow normally for 5 or 6 days, then fail to gain weight at the normal rate. The growth increment decreases slowly at first and by the 12th or 13th days there may even be a loss in weight. The rate of weight increase shows no correlation with the dose of estrogen administered. On the 14th day the weights of the young of injected mothers ranged from 11 to 17 gm. as compared

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with 26.5 gm. for those of untreated controls. The authors feel that the failure of the young to grow at the normal rate is not due to a suppression of lactation. In all of the mother rats milk exuded from the cut surfaces of the mammary glands at autopsy and sections showed alveoli distended with secretion (fig. 1, 2). Milk was present in the stomachs of most of the young when they were autopsied.

Urogenital Organs of Young

a), *Females: Ovaries.* Although no ovarian weights were taken at autopsy, histological examination showed that in the diethylstilbestrol-treated group development was inhibited in the young. The ovary of a normal 14-day-old rat contains many young follicles with granulosa 4 to 5 cell layers thick, and in some the beginning of antrum formation is visible. The ovaries of the suckling rats of mothers receiving the heaviest doses of diethylstilbestrol had developed only slightly beyond the sex cord stage and no follicles had a granulosa more than 2 to 3 cell layers in thickness. There was no indication of antrum formation. In the theelin-treated group, inhibition was apparent only in the young of the female receiving .5 mg. daily. Here also, no antrum

TABLE 1. VARIATIONS IN THE DIAMETERS OF UTERI OF 14-DAY-OLD FEMALE RATS OF MOTHERS INJECTED WITH VARYING AMOUNTS OF ESTROGEN DAILY

Hormone	Daily Dose, Milligrams	Diameters of Uteri of 14-Day-Old Young, mm.
Control		0.622
Theelin	.025	0.422
Theelin	.050	0.710
Theelin	.100	0.721
Theelin	.500	0.830
Diethylstilbestrol	.025	0.899
Diethylstilbestrol	.050	0.983
Diethylstilbestrol	.100	1.053

formation was seen, but the degree of inhibition did not approach that obtained with diethylstilbestrol.

Fallopian tubes. An inhibition of development of the Fallopian tubes occurred in the young of rats under the influence of diethylstilbestrol and in those of mothers under the heaviest dose of theelin. The diameter of the lumen was greatly reduced and the muscular wall thickened. The tubal epithelium was hyperplastic and showed evidence of vacuolization in the young of rats under the heaviest doses of each hormone.

Uteri. The diameters of sections of the uteri were obtained with an ocular micrometer. Two diameters at right angles were measured and averaged (table 1). With one exception, the diameters were greater in the experimental animals as compared with the controls. The greater the quantity of hormone administered, the greater the response. A given dose of diethylstilbestrol was more potent than the same amount of theelin.

The uterus of a normal 14-day-old rat shows well-differentiated myometrium and endometrium. The endometrium is lined with a simple columnar epithelium and uterine glands extend to its basal portion. The uteri of suckling rats from mothers receiving the three smaller doses of theelin showed no significant histological differences from the controls. The uteri of the young of those under the influence of the largest dose of theelin (.5 mg. daily) and those under the influence of diethylstilbestrol showed striking changes. The myometrium became thickened. Especially marked was

the hyperplasia of the circular muscle layer. Even more striking was the atypical character of the lining epithelium. There was a definite indication of stratification; the cells were large and in certain areas developed vacuoles of such a size as to give a pseudoglandular picture (fig. 13–20). The injected estrogens interfered with the normal development of uterine glands in the young. No glands were present in the young whose mothers were injected with the heaviest doses of diethylstilbestrol.

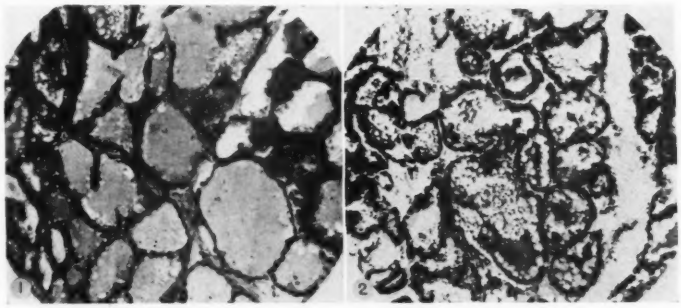


Fig. 1. MAMMARY GLAND OF D810 INJECTED WITH .1 mg. of theelin daily for 13 days beginning on day of parturition. Autopsied on 14th day. Fig. 2. MAMMARY GLAND OF D731 INJECTED WITH .1 mg. of diethylstilbestrol daily for 13 days beginning on day of parturition. Autopsied on 14th day.

The others showed various degrees of glandular development in inverse proportion to the dose of estrogen administered.

In the young of the females injected with .1 mg. of diethylstilbestrol the uterine epithelium was slightly stimulated as early as the 2nd day. By the 4th day the cells had increased markedly in height and the myometrium was differentiated and thickened. On the 6th day the epithelium had become atypical and the myometrium excessively developed. On the same day in the young of the theelin-treated group the myometrium was just beginning to differentiate and the epithelium did not differ from that in the controls which showed only the slightest indication of myometrial differentiation. The diameters of the uteri clearly showed the degree of stimulation (table 2).

Vaginae. When large doses of estrogen were injected into lactating rats beginning on the day of parturition, the vulvae of the suckling females appeared congested and swollen a few days after injections were begin. In several, the vaginal orifice opened as early as the 9th day and a typical estrous smear could be obtained if the vagina were gently flushed with a finely drawn pipette.

TABLE 2. DIAMETERS OF UTERI OF YOUNG FEMALE RATS OF MOTHERS INJECTED WITH .1 MG. OF THEELIN OR DIETHYLSTILBESTROL DAILY

Age in Days	Controls	Theelin-Treated Group	Diethylstilbestrol Treated Group
2	.216 mm.	.255 mm.	0.329 mm.
4	.324 mm.	.375 mm.	0.454 mm.
6	.341 mm.	.483 mm.	0.676 mm.
8	.397 mm.	.591 mm.	0.789 mm.
10	.471 mm.	.551 mm.	0.914 mm.
12	.500 mm.	.727 mm.	0.835 mm.
14	.622 mm.	.721 mm.	1.053 mm.

Sections of the vaginae of the 14-day-old young, cut at a level just below the neck of the bladder, were compared. This portion of the vagina, even in newborn young, has a well-established lumen. A decided stimulation was observed in the suckling young, directly correlated with the dose of hormone administered to the mother and

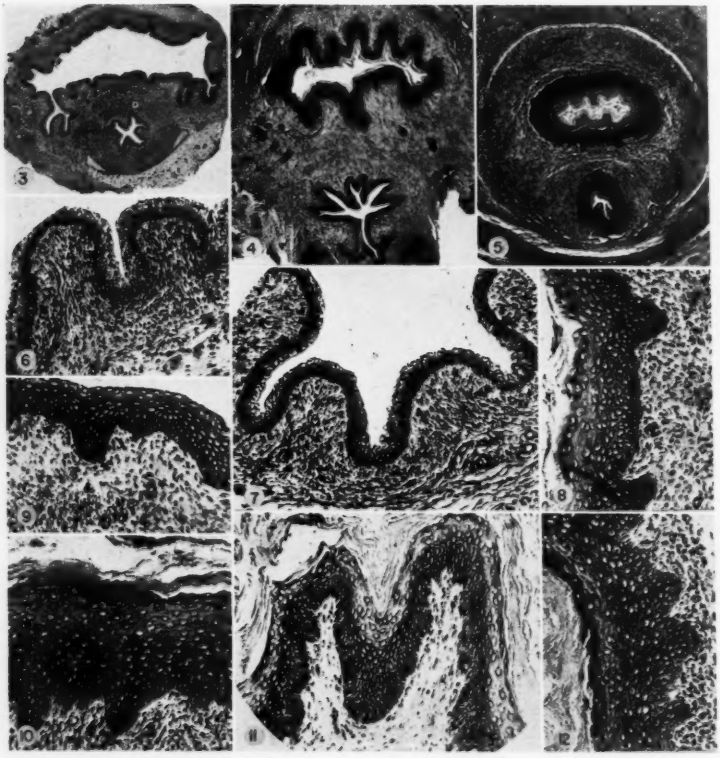


Fig. 3–12. VAGINAL EPITHELIUM OF 14-DAY-OLD RATS.

Fig. 3. UPPER VAGINA OF NORMAL RAT. Fig. 4. LOWER VAGINA; mother injected with .1 mg. of theelin daily beginning on day of parturition. Fig. 5. LOWER VAGINA; mother injected with .1 mg. of diethylstilbestrol daily beginning on day of parturition.

Fig. 6. EPITHELIUM OF UPPER VAGINA OF NORMAL RAT. Fig. 7. EPITHELIUM; mother injected with .025 mg. of theelin daily beginning on day of parturition. Fig. 8. EPITHELIUM; mother injected with .025 mg. of diethylstilbestrol daily beginning on day of parturition.

Fig. 9. EPITHELIUM; mother injected with .05 mg. of theelin daily beginning on day of parturition. Fig. 10. EPITHELIUM; mother was injected with .05 mg. of diethylstilbestrol daily beginning on day of parturition. Fig. 11. EPITHELIUM; mother injected with .1 mg. of theelin daily beginning on day of parturition. Fig. 12. EPITHELIUM; mother was injected daily with .1 mg. of diethylstilbestrol beginning on day of parturition.

with the potency of the hormone (fig. 3–12). Thus, in the offspring of the theelin-injected group, the vaginae showed little or no stimulation with a dose of .025 mg.; a moderate degree with .05 mg.; and great stimulation, with cornification equal to that in an adult in full estrus, with .1 mg. The young of the female given .5 mg showed intense cornification but the vaginal epithelium was atypical; it did not show

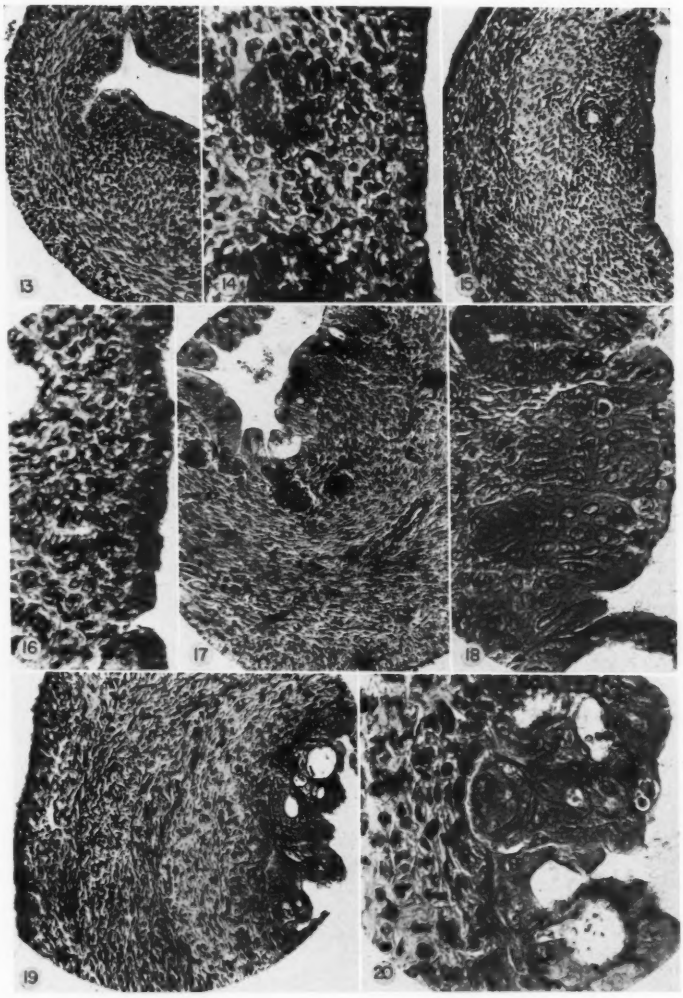


Fig. 13–20. Uteri and uterine epithelium of 14-day-old rats.

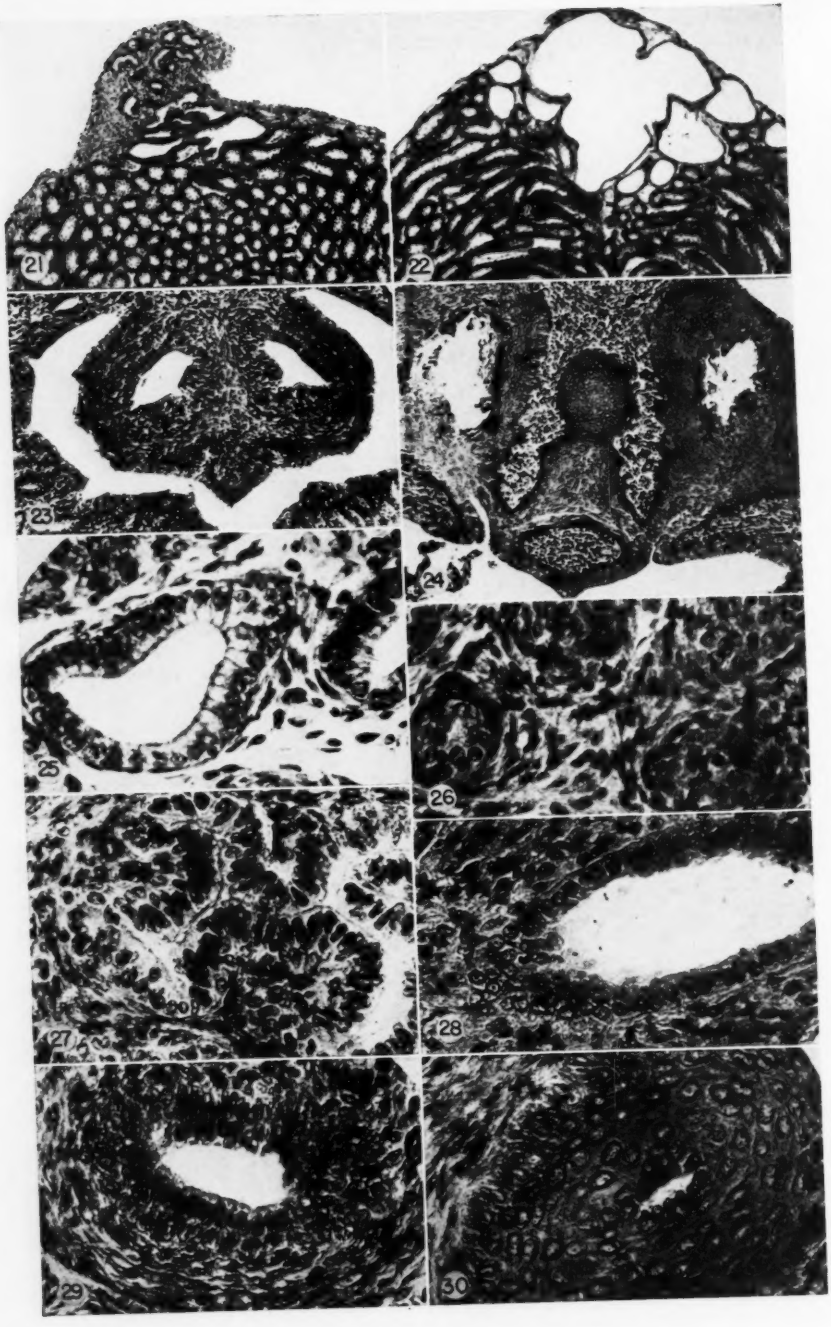
Fig. 13. Uterus of normal rat. Fig. 14. Epithelium of uterus in fig. 13, high power. Fig. 15. Mother injected with .1 mg. theelin daily beginning on day of parturition.

Fig. 16. Epithelium of uterus in fig. 15, high power. Fig. 17. Mother injected with .5 mg. of theelin daily beginning on day of parturition. Fig. 18. Epithelium of uterus in fig. 17, high power.

Fig. 19. Mother injected with .1 mg. of diethylstilbestrol daily beginning on day of parturition.

Fig. 20. Epithelium of uterus in fig. 19, high power.

the characteristic folds seen in the controls and in those animals under the influence of smaller amounts of theelin. The young of the diethylstilbestrol-injected rats showed even more striking effects. Here, the lowest dose (.025 mg.) resulted in definite cornification. The vaginal epithelium in this group, as in those under the heaviest dose of theelin, did not show the usual folding. Instead, the lining was smooth and the lumen small (fig. 3–12).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As early as the 2nd day of life the young of the mothers injected with .1 mg. of theelin showed a slight stimulation of the vaginal epithelium. This stimulation was progressive as time went on. Those under the influence of diethylstilbestrol were more advanced than those under the influence of theelin on the 4th day of life when the vaginal epithelium was 5 to 6 cell layers thick; the young of the theelin-treated group had 3 to 4 cell layers; and the controls, 2 to 3. In the offspring of rats of the diethylstilbestrol-treated group, cornification of the vaginal epithelium began between *days 8* and *10;* those of the theelin-treated group between *days 12* and *14*.

Bladders and urethrae. No differences were noted between the bladders and ureters of the experimental animals and the controls. The heaviest dose of diethylstilbestrol brought about a marked cornification of the epithelium at the distal end of the urethra in the suckling young. This was so intense as to make it somewhat difficult to distinguish vagina from urethra in this region. These females showed the partially hypospadic condition reported by Hain (1, 2, 3) and Greene (4).

TABLE 3. MEASUREMENTS OF TESTES OF 14-DAY-OLD MALE RATS OF MOTHERS INJECTED WITH VARYING AMOUNTS OF ESTROGEN DAILY

Hormone	Daily Dose, mg.	Measurements of Testes of 14-Day-Old-Young, mm.		
		Length	Width	Thickness
Control		5.4	3.4	3.1
Theelin	.025	4.4	3.0	2.8
Theelin	.050	4.3	2.6	2.5
Theelin	.100	5.1	3.1	2.9
Theelin	.500	4.3	2.8	2.4
Diethylstilbestrol	.025	3.9	2.7	2.5
Diethylstilbestrol	.050	3.9	2.6	2.4
Diethylstilbestrol	.100	3.4	2.8	2.4

b). *Males. Testes.* The length, width and thickness of the testes of the 14-day-old young were measured with a Vernier caliper under a binocular dissecting microscope after they had been fixed and dehydrated to 80 per cent alcohol. The dimensions of the testes of the animals under the influence of diethylstilbestrol were smaller than those under theelin, but the effects of various dosage levels were not obvious (table 3).

No significant histological differences were noted between the testes of suckling rats in the theelin-treated group and those of the controls. The seminiferous tubules in the young of the diethylstilbestrol-treated group were smaller in diameter and length, but the progress of spermatogenesis was not conspicuously retarded. A marked change occurred in the rete tubules which were enormously enlarged. The epithelial

Fig. 21–30. MALE REPRODUCTIVE TRACT OF 14-DAY-OLD RATS.

Fig. 21. TESTIS AND RETE TUBULES of normal 14-day-old rat. Fig. 22. TESTIS AND RETE TUBULES of 14-day-old rat; mother injected with .1 mg. of diethylstilbestrol daily beginning on day of parturition.

Fig. 23. MÜLLER'S TUBERCLE of normal 14-day-old male rat. Fig. 24. MÜLLER'S TUBERCLE OF 14-DAY OLD MALE RAT; mother injected with .1 mg. of diethylstilbestrol daily beginning on day of parturition.

Fig. 25. VENTRAL LOBE OF PROSTATE GLAND OF NORMAL 14-DAY-OLD RAT. Fig. 26. VENTRAL LOBE OF PROSTATE GLAND OF 14-DAY-OLD RAT; mother injected with .1 mg. of diethylstilbestrol daily beginning on day of parturition.

Fig. 27. TIP OF SEMINAL VESICLE OF NORMAL 14-DAY-OLD RAT. Fig. 28. TIP OF SEMINAL VESICLE OF 14-DAY-OLD RAT; mother injected with .1 mg. of diethylstilbestrol daily beginning on day of parturition.

Fig. 29. DUCT OF COWPER'S GLAND OF NORMAL 14-DAY-OLD RAT. Fig. 30. DUCT OF COWPER'S GLAND OF 14-DAY-OLD MALE RAT; mother injected with .1 mg. of diethylstilbestrol daily beginning on day of parturition.

lining was flattened and the long axis of the nuclei ran parallel to the basement membrane rather than vertically as in the controls (fig. 21 and 22). No variations were observed in the interstitial tissue.

When .1 mg. of diethylstilbestrol was administered daily to the mothers the effects on testes and rete tubules were apparent by the 6th to 8th days (table 4).

Epididymis. The cells lining the epididymis of the normal 14-day-old rat have basal nuclei and an epithelial height approximately twice the nuclear diameter. The epididymides of suckling rats in the theelin-treated group resemble those in the control groups, but those in the diethylstilbestrol-treated group had an epithelial height

TABLE 4. MEASUREMENTS OF TESTES OF YOUNG MALE RATS OF MOTHERS INJECTED DAILY WITH .1 MG. OF THEELIN OR DIETHYLSTILBESTROL

Age in Days	Control Group	Theelin-Injected	Diethylstilbestrol
2	1.9X1.6X1.4	2.0X1.6X1.5	1.9X1.5X1.3
4	2.5X2.0X1.9	2.5X2.1X1.8	2.4X1.9X1.8
6	3.0X2.3X2.2	3.0X2.1X1.9	2.5X2.0X1.8
8	3.7X2.5X2.4	3.6X2.3X2.2	2.7X2.0X1.9
10	3.9X2.7X2.5	4.2X2.6X2.5	2.8X2.5X2.2
12	4.7X3.0X2.9	4.6X2.8X2.5	3.3X2.6X2.3
14	5.4X3.4X3.1	5.1X3.1X2.9	3.4X2.8X2.4

Measurements given in millimeters.

equal to only one nuclear diameter. This effect produced by diethylstilbestrol was evident on the 6th day of life.

Ductus deferens. Comparable sections of the ductus deferens at the level of the caput epididymis showed that in the young of the diethylstilbestrol-treated group the external diameter and the diameter of the lumen were smaller than in those of the theelin-treated and control groups. In the former, the epithelium was also retarded in development and the stereocilia extending into the lumen were less numerous.

Seminal vesicle. In the normal 14-day-old rat each seminal vesicle contains a central lumen with lateral ramifying branches extending throughout the length of the gland. The epithelium is columnar, the cells being slightly longer than the nuclei. The seminal vesicles of the young of the theelin-injected rats showed no deviation from normal but those of the diethylstilbestrol-injected group showed an inhibition of development proportional to the dose administered. When .1 mg. of diethylstilbestrol was injected daily the epithelium was low and no branching of the lumen was evident (fig. 27 and 28).

Prostate gland. Only the ventral prostatic lobes were examined in detail. In the normal 14-day-old rat the acini have clearly defined lumina and the epithelium in places shows the 'light areas' between nucleus and lumen characteristic of the adult gland. None of the suckling males in the theeelin-treated group differed from the normal. A marked inhibition, however, was observed in the diethylstilbestrol-treated group, an effect again correlated with the dose administered. Those animals whose mothers received the greatest dose of diethylstilbestrol (.1 mg. daily) had no lumina in the acini (fig. 25 and 26).

Urogenital sinus. Serial sections of the entire urogenital sinus were examined. The prostatic urethra and the prostatic utricle (vagina masculina) of the normal 14-day-old rat are lined with transitional epithelium. The suckling males of the theelin-injected group showed no modification from normal. The change in the young of the

diethylstilbestrol-treated group, however, was striking (fig. 23 and 24). The epithelium was cornified and the degree of stimulation was correlated with the dose administered. In the males to the mother of which was given .1 mg. of diethylstilbestrol daily, cornification of the epithelium of the ejaculatory ducts and prostatic utricle was so intense as to practically obliterate the lumen. The cornified area extended up the ejaculatory ducts to the point of junction of seminal vesicle and ductus deferens. In the urogenital sinus proper only the Müllerian tubercle was affected. Cornification of the anterior and lateral walls was not observed. On the posterior wall, the cornified area extended proximally to the openings of the ejaculatory ducts; distally it extended only a short distance.

With the large dose of diethylstilbestrol administered to the lactating mothers the epithelium of the ejaculatory ducts, prostatic utricle and contiguous portion of the urogenital sinus gave a response in the suckling male paralleling that of the suckling female, a definite stimulation being apparent by the 4th day and cornification occurring between *days* 6 and 8.

The bladder, ureters and remainder of the urethra were not affected by the injections.

Cowper's gland. In the normal 14-day-old rat the acini of Cowper's gland are clearly differentiated and the epithelial cells well-developed. A small duct leads to the urethra on each side a considerable distance below the openings of the ejaculatory ducts and prostatic utricle. A median dorsal diverticulum with small ramifying branches leads into the urethra at the point where the ducts of Cowper's glands enter. In the young of rats subjected to the heaviest doses of diethylstilbestrol there was an inhibition in development of Cowper's gland and of the dorsal urethral diverticulum. The epithelium lining the ducts of these glands and the diverticulum was highly stratified (fig. 29, 30). This was apparent by the 8th to 10th days. The heaviest dose of theelin caused a slight inhibition in development of Cowper's gland but no stratification of the duct epithelium.

DISCUSSION

In the present experiment the failure of the young of the estrogen-treated mothers to grow at the normal rate is of interest since the maternal mammary glands even on the 14th day contain an abundance of secretion, indicating that lactation is not suppressed. Although most investigators (5–17) are of the opinion that large amounts of estrogen interfere with the endocrine balance necessary for normal lactation the authors feel that a loss of maternal instinct (2, 18) is primarily responsible for the failure of the young to grow at the normal rate.

The behavior of the mother rats becomes abnormal the 6th or 7th day after injections of estrogens are begun. Instead of showing the usual solicitude for the young, their care becomes sporadic. At times the pups are scattered about the cage and are cold. The intervals of care become less and less frequent as the period of injection continues. It is thus possible that inhibition of growth can be attributed, at least in part, to a lack of opportunity to suckle rather than failure of the mammary glands to secrete. It is known that withdrawal of the young or cessation of the suckling stimulus will cause a rapid retrogression of the mammary glands. Failure of the mother to permit the young to suckle may then be the direct cause of mammary gland failure and the injected estrogen is only indirectly responsible. Caution should be exercised in interpreting failure of lactation on the basis of failure of the young to grow normally.

Another factor which may account, in part, for the smaller size of the offspring

of estrogen-treated rats is the inhibition in production of growth hormone from their hypophyses (19).

In the rat the vaginal orifice is not normally established until the time of puberty which occurs between the 40th and 90th days of life (20). Although reports of others (2, 21–23) show that the vaginae of immature rats and mice may be directly or indirectly stimulated by estrogen, in no case has it been demonstrated that a response can be obtained as early as our experiments indicate. The vaginal epithelium responds to estrogens as early as the 3rd or 4th day of life; cornification may appear by the 6th to 8th days; and the vaginal orifice may be established by the 9th day.

The absence of the characteristic folding in the vaginae of animals under the influence of the heaviest doses of estrogen may be due to a very intense stimulation of the vaginal epithelium and simultaneous suppression of body growth.

Myometrial hypertrophy, and hyperplasia of the myometrium and endometrium, may be induced in the uteri of adult castrate rats by prolonged estrogen injection (23–33). The stimulation of the myometrium and endometrium in the young rats reported here is of special interest in view of the early age at which it became apparent and the short time during which the animals were treated.

The intense stimulation of the epithelium of the ejaculatory ducts, prostatic utricle and Müllerian tubercle in the males of mothers which were injected with diethylstilbestrol, emphasizes the embryological homologies of this region. It is a general conception that the ejaculatory ducts of the male are Wolffian duct derivatives. If we interpret the observations reported here on an embryological basis, it would seem that the lower Müllerian ducts may contribute to the formation of the ejaculatory ducts since under the influence of diethylstilbestrol they show such a pronounced stratification and cornification, a reaction not observed in Wolffian duct derivatives. The reason for the response of the ducts of Cowper's glands and of the median dorsal urethral diverticulum is not clear.

Although the testes of animals from mothers receiving diethylstilbestrol were smaller than those of the controls, this may be only a reflection of the general suppression of body growth. The significance of the unusual variation observed in the rete tubules is not obvious.

On the basis of our findings it is apparent that at birth the differentiation of the urogenital systems of both male and female offspring has progressed beyond the point where intersexual modifications can be produced by administering estrogens (1, 3, 4, 35–38). No Wolffian duct derivatives persist in the females and no nipples develop in the males. Only the external genitalia of the females seem to be incompletely differentiated at birth since estrogens administered after birth are capable of producing a permanent partial hypospadias (1–4). It is our belief that the cornification of the urethral epithelium of the females reported here is correlated with this anomalous development of the external genital region.

There are several ways by which the young of estrogen-injected females may receive the hormone. It is possible that the estrogen excreted by the mother may be licked off of the body or absorbed through the skin. The more probable condition is its direct transmission through the mothers' milk. The actual occurrence of estrogen in milk under normal and experimental conditions has been demonstrated (1, 2, 39, 40).

Estrogens are less effective when orally administered than when injected. A uniform rate of absorbtion is more effective than the hormone sporadically available to the organism. Since the stomachs of suckling rats contain milk at practically all times, any hormones contained in the milk are undoubtedly being passed into the

blood stream at a practically uniform rate. Hence, when administered in the manner described they would be more effective than if they were given by other means. This difference in method may account for the results obtained here which differ from reports of other workers who injected estrogen directly.

That diethylstilbestrol is more potent than natural estrogens has been shown by many investigators. It is not surprising, then, that a daily dose of .1 mg. given to the mother exerts a more profound effect on the offspring than a corresponding amount of theelin. Natural estrogens are inactivated by the liver and diethylstilbestrol is not (41). Since hormones received by these young pass from the alimentary canal via the portal vein to the liver, it is to be expected that the effects obtained with diethylstilbestrol should be more pronounced than those obtained with theelin. Even when a dose of theelin (.5 mg. daily) which is roughly comparable to a dose of .1 mg. of diethyl stilbestrol was administered, the effects obtained were not so marked.

SUMMARY

Estrogens injected into lactating rats, beginning on the day of parturition, have a pronounced effect upon the growth of the young and upon the development of their urogenital systems. The young grow normally for 5 or 6 days and then fail to gain weight at the normal rate. Lactation does not seem to be suppressed in the mothers. Loss of maternal instinct and neglect of the young probably account in part for their failure to grow normally.

In general, the influence on the urogenital systems is proportional to the dose administered to the mother and diethylstilbestrol is much more effective than an equal amount of theelin. In the female young under the influence of the heaviest doses of estrogens there is on the 14th day, an intense cornification of the vaginal epithelium; an atypical pseudostratified uterine epithelium and hyperplastic myometrium; a suppression of uterine gland, Fallopian tube and ovarian development. In the males the testes are small and the development of the accessory sex organs inhibited. There is an abnormal development of the rete tubules, intense epithelial cornification of the ejaculatory ducts and prostatic utricle, and marked stratification of the ducts of Cowper's glands.

The influence of the hormones can be observed as early as *day* 4. In some females the vaginal orifice is established as early as *day* 9.

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EFFECT OF THE ESTROUS CYCLE ON THE ACTION OF TESTOSTERONE PROPIONATE ON THE ORGAN AND BODY WEIGHTS OF FEMALE RATS[1]

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IN A PREVIOUS COMMUNICATION (1) from this laboratory, it was reported that the ultimate effect of testosterone propionate on the primary and secondary sex organs of the female rat depended upon the presence or absence of recent corpora lutea at the time of the first injection. Thus, it was found possible to produce two entirely different sets of responses in the animals, depending on whether the male hormone was first administered to *a*) adults at estrus or *b*) to immature animals or to adults at diestrus.

As only the primary and secondary sex organs were considered in the report mentioned above, a repetition of the experiment appeared to be indicated for the following reasons. *a*). To confirm the uniformity with which the changes could be reproduced, and *b*), to extend the investigation to include certain other organs, namely, the adrenal, kidney, liver and thyroid. The problem discussed here deals with body and organ weight changes occuring in female rats following treatment with testosterone propionate begun both at estrus and at diestrus.

Many studies on weight changes already have been made (2–13) but the importance of the ovarian factor does not seem to us to have been sufficiently stressed. We will thus show that the condition of the ovary at the time of the first injection of the hormone has a definite influence on the body weight as well as on some of the organ weights.

PROCEDURE

The vaginal estrous cycle of a number of normal female rats approximately 82 days old was followed for a period of 28 days, after which 56 animals, whose cycles were regular and within normal limits were selected for use. These were divided into four groups of 14 each. Thus, at the beginning of the investigation they were approximately 110 days old. The animals were of the Stanford strain, and had been fed Tioga Chow. They had been housed not more than four to a cage, and had been permitted tap water ad libitum.

The preparations used in the experiment were *a*), testosterone propionate[2] dissolved in sesame oil (1.0 cc. = 10.0 mg.) and *b*), for the control groups, sesame oil alone. When the experiment was originally set up it was planned to inject standardized doses of 0.4 cc. of one or the other of these preparations every alternate day over

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[2] The testosterone propionate was supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

a period of 20 days, thus giving a total dose of 40.0 mg. of testosterone in sesame oil or 4.0 cc. of sesame oil alone.

Owing, however, to unexpected and unavoidable experimental considerations, we were forced to shorten the duration of treatment in some cases to 16 days, hence the original plan of administration every other day was, of necessity, abandoned. In those cases, however, where the duration of treatment was less than 20 days, larger doses were administered toward the end of the treatment period, so that in all cases the total dose originally planned was given. There is no reason to assume that this procedure had any untoward consequences on the results, as the principal biologic effect of treatment (i.e., the maintenance and hypertrophy of the corpora lutea) is already reached after the administration of 20 mg. of testosterone, which is but half of the total dose provided. Thus, in the following details of group treatments, injections on alternate days for at least 8 injections should be understood.

Group I. Sesame oil at estrus (*SE*). Received 4.0 cc. of sesame oil subcutaneously in divided doses. First injection at late estrus.

Group II. Sesame oil at diestrus (*SD*). Received 4.0 cc. of sesame oil subcutaneously in divided doses. First injection on second day of diestrus.

Group III. Testosterone at estrus (*TE*). Received 40.0 mg. (4.0 cc.) of testosterone propionate in sesame oil subcutaneously in divided doses. First injection at late estrus.

Group IV. Testosterone at diestrus (*TD*). Received 40.0 mg. (4.0 cc.) of testosterone propionate in sesame oil subcutaneously in divided doses. First injection on second day of diestrus.

The animals were killed after periods varying from 16 to 21 days, as follows. Four rats of each group in 16 days; 4 rats of each group in 18 days; 6 rats each of *Groups II* and *IV* in 20 days, and 6 rats each of *Groups I* and *III* in 21 days. All were killed 24 hours after the last injection except the 6 animals of *Groups I* and *III* respectively, in which the time elapsing between the last injection and the post-mortem examination was 48 hours.

In order to obtain comparable weights a uniform autopsy procedure was instituted. Under ether anesthesia the animal was bled to death by heart puncture. The organs were then removed in the following sequence: thyroid, liver, kidneys, adrenals, ovaries and uterus. The thyroids, adrenals, ovaries and uteri were weighed on a torsion balance to .001 gram. It should be noted that the uteri were weighed in a dry state, the organ being split longitudinally and the fluid removed with blotting paper.

ANALYSIS OF DATA

The mean body weight of the 56 animals was 157.5 gm. at the start of the investigation, with a standard deviation of 12.5 gm., the limits at the 5 per cent point thus being 132.5 and 182.5 gm. All animals except one (which was included although being just above the upper limit) were within this range; thus the sample was considered homogeneous from the point of view of starting weight.

The usual method of evaluating the significance of the differences between the means of the treated and untreated organ weights as developed by Fisher (14) could not properly be applied to the data until certain preliminary steps had been taken.

As is well known, Fisher's method is based upon the following statistics: the numbers in the samples (N) with their corresponding degrees of freedom (n), the difference between the means to be tested ($\bar{y}_2 - \bar{y}_1$), and the standard error of this difference, $s_{(\bar{y}_2 - \bar{y}_1)}$. The statistical significance of the difference between the means is determined by calculating a value

$$t = \frac{\bar{y}_2 - \bar{y}_1}{\left[\left(\frac{s_{y_1}}{\sqrt{N_1}}\right)^2 + \left(\frac{s_{y_2}}{\sqrt{N_2}}\right)^2\right]^{1/2}}$$

with which to enter a table of probabilities, taking into account the proper number of degrees of freedom. The usual standard of significance is $P < .05$ which is to be interpreted as follows. In a random sample, only 5 per cent of the cases would show an observed difference as great or greater than that found, as the result of chance alone.

In this investigation, however, the picture was complicated by the fact that the body weights of the animals were not constant; thus the organ weights, which are in part at least a function of body weight, could not be used in their raw state until a proper correction had been made. Besides, several of the groups disclosed an occasional animal (never more than one) whose organ weight appeared to deviate so far from that of its fellows as to suggest that it be rejected from the sample.

Thus, in order to prepare the data for the application of Fisher's method, the following steps were taken. In order to eliminate that part of the variation in organ weight due to variation in body weight, regression equations were calculated for each organ in each group. Thus it was possible to measure the latter variation and reduce it to statistical control (15). In other words, the standard error of estimate (s_e) was substituted for the standard deviation of the sample, the former thus expressing the variation in organ weight after the accompanying variation in body weight had been excluded. Furthermore, instead of using the raw organ weight means of the original arrays, the mean body weight of the two groups to be compared was calculated, and from this figure the estimated value of the mean organ weight (E) was determined from the corresponding regression equations.

It should be noted that in the predicting equation, $E = a - b\bar{x}$, E plays the part of the mean, the standard errors of estimate being the actual deviations from this mean.

For example, the E values for the comparison of the *SE* and *SD* groups of the adrenal were calculated in the following manner from the regression equations (which equations are not being published here).

Mean x value for *SE* and *SD*:

$$\frac{\Sigma(x) = 2329 + 2059 = 4388}{N = \quad 14 + \quad 13 = \quad 27} = 162.52 = \bar{x}$$

$$E_2 \text{ (i.e., for } SD) = -6.21 + .3399(162.52) = \quad 49.03$$
$$E_1 \text{ (i.e., for } SE) = -6.27 + .3601(162.52) = \quad 52.25$$
$$E_2 - E_1 = -3.22$$

By this transformation,

$$t = \frac{E_2 - E_1}{\left[\left(\frac{s_{e_1}}{\sqrt{N_1}}\right)^2 + \left(\frac{s_{e_2}}{\sqrt{N_2}}\right)^2\right]^{1/2}}$$

which, being in all respects analogous to the expression given previously, is a more accurate exposition of the meaning of the data.

Following the calculation of all regression equations, the individual organ weights from which they were derived were scrutinized, and those deviating from the regres-

TABLE 1. STATISTICAL ANALYSIS OF EFFECTS OF TESTOSTERONE PROPIONATE IN FEMALE RATS

Statistic	SE	SD	TE	TD	SE:SD	S	TE:TD	S:TE	S:TD
					Adrenal, weight in mg.				
N	14	13	13	14	27	27	27	40	41
$\bar{x}$	166.36	158.38	167.85	170.29	162.52	162.52	169.11	164.25	165.17
$\bar{y}$	53.64	47.62	46.69	37.57		50.74			
s_y	8.5799	6.0964	4.6637	4.7312		7.9614			
s_e	6.8581	4.6851	3.6554	3.6243		5.8999			
(E_2-E_1)					−3.22		−9.64	−5.50	−15.28
s_d					2.2468		1.4021	1.5221	1.4924
t					1.433		6.875	3.613	10.239
n					25		25	38	39
P					0.2		0.001	0.001	0.001
					Liver, weight in gm.				
N	13	13	13	14	26	26	27	39	40
$\bar{x}$	166.46	157.92	169.54	170.29	162.19	162.19	169.93	164.64	165.02
$\bar{y}$	8.0646	7.7646	8.2200	7.5614		7.9146			
s_y	.7913	.7623	.7066	.9431		.7765			
s_e	.6148	.7826	.3370	.8267		.7436			
(E_2-E_1)					−.20		−.69	+.06	−.59
s_d					.2760		.2398	.1732	.2647
t					.725		2.877	.346	2.229
n					24.		25.	37.	38.
P					.5		.01	.7	.035
					Ovary, weight in mg.				
N	13	13	14	14	26	26	28	40	40
$\bar{x}$	165.85	158.31	169.43	170.29	162.08	162.08	169.86	164.65	164.95
$\bar{y}$	55.77	50.54	64.00	25.00		53.15			
s_y	3.7528	6.0690	12.8482	4.6740		5.6107			
s_e	2.8938	6.3360	12.6265	4.5947		5.2984			
(E_2-E_1)					+1.26		−39.17	+9.11	−29.12
s_d					1.9319		3.5910	3.5309	1.6086
t					.652		10.908	2.580	18.103
n					24.		26.	38.	38.
P					.5		.001	.02	.001
					Thyroid, weight in mg.				
N	14	13	13	14	27	27	27	40	41
$\bar{x}$	166.36	158.38	169.54	170.29	162.52	162.52	169.93	164.80	165.17
$\bar{y}$	13.43	14.15	16.62	14.21		13.78			
s_y	2.4651	1.1547	2.3274	2.8284		1.9513			
s_e	2.4244	1.1782	1.8447	1.8358		1.9763			
(E_2-E_1)					+.84		−2.50	+2.35	−.36
s_d					.7257		.7088	.6375	.6207
t					1.158		3.527	3.686	.580
n					25.		25.	38.	39.
P					.3		.01	.001	.6
					Uterus, weight in mg.				
N	14	14	14	14	28	28	28	42	42
$\bar{x}$	166.36	158.21	169.43	170.29	162.29	162.29	169.86	164.67	164.95
$\bar{y}$	401.14	376.14	775.21	424.07		388.64			
s_y	47.2066	126.8051	191.5331	42.2948		94.7470			
s_e	41.7212	130.0101	197.1798	43.9961		92.9348			
(E_2-E_1)					−10.73		−351.90	+373.21	+31.04
s_d					36.4916		53.9938	55.5475	21.1357
t					.294		6.517	6.719	1.469
n					26.		26.	40.	40.
P					.8		.001	.001	.15

N=number in group; $\bar{x}$=mean body weight (gm.); $\bar{y}$=mean organ weight (gm. or mg. as indicated); s_y=standard deviation of $\bar{y}$; s_e=standard error of estimate; (E_2-E_1)=difference between the predicted mean values of y; s_d=standard error of this difference; $t=(E_2-E_1)/s_d$; n=degrees of freedom; P=probability.

Note that occasionally s_e is greater than s_y. This is due to the additional degree of freedom lost in the calculation of the former, and is unimportant here.

sion line by more than $\pm 2s_e$ were discarded. A new regression equation was then calculated.

The significance of the differences between the various estimated means were now

determined according to Fisher's method as described, and the probabilities computed. The differences were tested in the following order for each of the organs under consideration.

a). The sesame oil controls, *SE* and *SD*.

b). If the difference between these means was not of a significant order, the groups were pooled to form the larger (and hence more reliable) control group, *S*. As was to have been expected, such a pooling was possible for all control organ weights.

c). The treated groups, *TE* and *TD*.

d). If the differences between these means were not of a significant order, the groups were pooled to form the larger treated group, *T*.

TABLE 2. STATISTICAL ANALYSIS OF EFFECT OF TESTOSTERONE PROPIONATE IN FEMALE RATS

Statistic	SE	SD	TE	TD	SE:SD	S	TE:TD	T	S:T
					Kidney, weight in gm.				
N	14	13	14	14	27	27	28	28	55
$\bar{x}$	166.36	158.46	169.43	170.29	162.56	162.56	169.86	169.86	166.27
$\bar{y}$	1.4179	1.3815	1.7264	1.6686		1.4004		1.6975	
s_y	.0874	.1020	.1513	.1304		.0949		.1418	
s_e	.0733	.0797	.1039	.1000		.0741		.1049	
(E_2-E_1)					−.001		−.06		+.25
s_d					.0295		.0385		.0244
t					.034		1.558		10.246
n					25.		26.		53.
P					.9		.2		.001
					Body weight, instantaneous growth rates				
								SE:TE	SD:TD
N	13	13	13	14	26		27	26	27
$\bar{x}$	164.08	152.77	159.31	154.93	158.42		157.04	161.69	153.89
$\bar{y}$	.0738	.1886	.3321	.5199					
s_y	.2129	.1901	.1792	.0907					
s_e	.2210	.1586	.1345	.0943					
(E_2-E_1)					+.1838		+.2140	+.2825	+.3161
s_d					.0755		.0450	.0718	.0507
t					2.434		4.756	3.935	6.235
n					24.		25.	24.	25.
P					.02		.001	.001	.001

See footnote to table 1.

e). If the treated groups differed significantly from each other, they were compared either with their corresponding controls or with the pooled control group.

The body weight changes were treated in a similar manner, except that the gain was expressed as an instantaneous percentage rate in order to eliminate the variation caused by *a*), the different starting weights and *b*), the differences in the duration of the experiment for the individual animals.

These instantaneous percentage rates were calculated according to the method described by Fisher (16). He states: "The relative growth rate at any instant is

$$\frac{1}{m}\frac{dm}{dt}=\frac{d}{dt}\log_e m$$

where m is the mass and t the time; and may be calculated from any two observations:

$$R=\frac{\log_e m_2-\log_e m_1}{t_2-t_1}$$

where the suffixes 1 and 2 indicate the first and second observations. This gives correctly the average value of the relative growth rate over the period between the two observations, whatever may be the nature of the changes in rate over this period." Multiplying the obtained value of R by 100 then gives the instantaneous percentage rate.

The pertinent statistics are given in tables 1 and 2.

Effects of Treatment

Testosterone propionate, when administered in the manner previously described, had the effects stated below. Only statistically significant results are reported. The mathematical probabilities may be read directly from the tables.

a). *On the adrenal.* The weight was decreased, both on administration at estrus and at diestrus, the effect, however, being more marked in the latter group.

b). *On the kidney.* The weight was increased, the phase of the estrous cycle at which administration took place having no bearing on the result.

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON ORGAN WEIGHTS

Phase of Cycle	Weight Changes	Adrenal	Kidney	Liver	Ovary	Thyroid	Uterus	Body Growth
Estrus	Increase		+		+	++	++	+
	No change			o				
	Decrease	+						
Diestrus	Increase		+					++
	No change					o	o	
	Decrease	++		++	++			

o = No change; + = significant change with respect to control; ++ = significantly greater effect.

c). *On the liver.* When administered at diestrus, the weight was decreased.

d). *On the ovary.* An increase in weight resulted on administration at estrus. Administered at diestrus, however, a decrease in weight was observed.

e). *On the thyroid.* Administration at estrus resulted in an increase in weight.

f). *On the uterus.* Administration at estrus resulted in an increase in the weight of the dried organ.

g). *On the body weight.* The instantaneous percentage rate of growth was increased in both the controls and the treated animals regardless of the phase of the cycle, but the effect was much greater when the testosterone was administered at diestrus. Specifically, the increase was of the order of 0.50 per cent per day for the diestrus, as compared with only 0.30 per cent per day for the estrous group.

The effects are summarized in table 3.

DISCUSSION

The literature reporting the action of male hormone on the female rat presents many contradictory statements concerning weight changes in the various organs. Such conflicting results may be due, in part at least, to a disregard, not only of the great variations normally present in all small laboratory animals, but also to the effect of the total body weight on the individual organs themselves. Thus, in addition to the application of statistical control to the data, we should like also to point out that *a*), the sequence of the removal and weighing of the organs was consistently adhered to, and *b*), a thorough knowledge of the estrous cycle was secured by taking daily vaginal

smears over a period of 4 weeks prior to the first injection. As a rule, all animals with cycles of 6 days or more were rejected.

The present investigation concerns itself only with physical (i.e., weight) changes effected by testosterone propionate, the morphological alterations having been already described (1).

In a recent symposium on androgen therapy Geist, Salmon and Hamblen (17–19) arbitrarily classified the effects of male hormone on the female as 'gynecomimetic,' 'antigynecogenic,' and 'arrhenomimetic.' By gynecomimetic, the authors understand effects which simulate the action of estrogens, progesterone, gonadotropins and mammotropins. Under anti-gynecogenic effect, the suppression of vaginal cornification, ovulation and follicle growth is considered, while arrhenomimetic action is defined as that simulating the male sex characteristics such as hypertrophy of the clitoris and preputial gland, male behavior, the growth of combs in female chicks, crowing in hens and singing in female canaries (18).

So far as the rat is concerned, we feel that the presence or absence of a responsive set of corpora lutea determines the predominant action of testosterone (1). When the hormone is given at estrus, histologic and weight studies suggest a predominantly gynecomimetic action, consisting of pregnancy-like changes in the ovaries, mammary glands, vaginae and thyroids. In addition, the uterus becomes hypertrophic. The enlargement of the kidneys, which are normally heavier in the male rat (20) and the decrease in weight of the adrenal gland might be considered arrhenomimetic responses, but it should be noted that the adrenal effect is less marked than appears below.

On the other hand, testosterone administered at diestrus resulted in smaller ovaries, low vaginal epithelium, and no gain in uterine weight, all of these effects being due to the lack of functioning corpora lutea. The kidneys increased, and the adrenals decreased in weight, the latter effect being much more marked than in the estrous group. In addition, the instantaneous percentage rate of gain in body weight was the largest encountered in the four groups. These effects appear to us to be predominantly of an arrhenomimetic nature.

Hence, the gynecomimetic and arrhenomimetic actions of testosterone just described appear to depend on the condition of the ovary at the time of the first administration.

We have failed to produce these changes in other similar experiments in 15 per cent of the animals (12 of 78) which were injected at what was presumably the proper time in each phase of the cycle. In every instance, however, we have traced back the cause of such failures to incorrect timing. For example, in the experiment published here, the first treatment was given at late estrus, i.e., approximately at the time of ovulation. Our estrous failures, mentioned above, were all included in those animals injected at metestrus (the stage of the young and most responsive corpora lutea). They are thus easily understood when consideration is taken of the time necessary (*a*), for the resorption of the hormone from the subcutaneous injection (*b*), for its action on the hypophysis (stimulation of the production of lactogenic hormone) and (*c*), for the action of the released pituitary lactogenic hormone upon the ovaries (maintenance of functional corpora lutea). That it is the lactogenic hormone which fulfills this task has been amply demonstrated by Evans, Simpson and Lyons (21).

In our present diestrous group, the second day of that phase was found most satisfactory for injection, the failures having been produced when injection was made on the first day. Occasional observations on rats which received the first treatment at proestrus, however, showed the same reaction as was seen in the diestrous group.

Since ovulation and corpus luteum formation would not have occurred for another 24 to 36 hours in such rats, sufficient time was allowed for a resorption of the testosterone and its subsequent action on the pituitary gland, thus preventing luteinization (1).

SUMMARY

Testosterone propionate influences the weight of female rat organs. When administered at estrus there is an increase in the weight of the kidneys, ovaries, thyroids and uteri, and a decrease in the weight of the adrenal. There is no effect on the liver. When administered at diestrus there is an increase in kidney weight of the same order as before, a decrease in liver weight, a more marked decrease in adrenal weight and a decrease in the weight of the ovary. The instantaneous percentage rate of body growth is increased both at estrus and diestrus, but the effect is much greater at diestrus.

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FURTHER OBSERVATIONS ON THE RELATIVE ABSORPTION RATES OF PELLETS OF VARIOUS CRYSTALLINE COMPOUNDS IMPLANTED SUBCUTANEOUSLY IN RATS

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THIS PAPER CONSTITUTES a second report on the relative absorption rates of uniformly-made pellets of various crystalline compounds which were implanted subcutaneously in rats. Also included is a consideration of the factor of crystal size in the absorption of pellets made in the author's apparatus.

MATERIALS AND METHODS

Study of relative absorption rates. The apparatus and technique for preparation, implantation, and recovery of the pellets has already been fully described (1). Briefly, the apparatus consists of a steel plate in which perforations form molds for cylindrical pellets. Uniform compression was achieved by subjecting the crystals in each mold to 35 blows from a 'pile driver,' the weight on the latter always being dropped from the same height. No excipient was used.

Fifteen pellets of desoxycorticosterone acetate, 8 of estriol, 15 of androsterone, 6 of α-estradiol, 9 of α-estradiol dipropionate, 15 of estrone, 13 of cholesterol, and 6 of α-estradiol benzoate, carefully weighed, were implanted in 56 male and 31 female sexually mature but not senile rats.[1,2] The implantations were made subcutaneously under ether anesthesia in the lower right abdominal quadrant. Each rat received one pellet. Every animal was weighed at the time of implantation.

After varying time intervals the rats were killed and reweighed. The pellets were removed from their capsules, dried to constant weight in a desiccator, and reweighed. The percentage absorption was then calculated and plotted against the number of days the pellet had been in situ (Fig. 1). For each compound, the number of days required for 90 percentage absorption was used as the basis of comparison.

Study of factor of crystal size. Using the same apparatus and technique, three groups of 10 pellets each were made from specially prepared, almost microscopic crystals of methyl testosterone, desoxycorticosterone acetate, and testosterone monopropionate. Three other groups of 9 or 10 pellets each were made from very large, coarse crystals of the same three compounds.[1] It was necessary to break the largest crystals before they could be introduced into the molds. Each of 29 male and 30 female rats received one pellet, implanted in the manner described above. The tech-

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[1] The author is indebted to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J., to Dr. Erwin Schwenk of the Schering Corp., Bloomfield, N. J., and to Dr. J. F. Biehn of the Abbott Laboratories, Chicago, Ill., for the compounds used in these experiments.

[2] The animals and facilities for carrying out some of the studies were generously provided by the Department of Biology of Swarthmore College.

nique already outlined was employed for the recovery of the pellets and the study of their weight loss.

All rats in both groups were maintained under essentially similar conditions.

RESULTS

Since the above studies, as well as those described in an earlier paper (1), were carried out under essentially uniform conditions and techniques for preparing and handling the pellets and maintaining the animals, it is believed that all of the relative absorption rates obtained are directly comparable.

Relative absorption rates. As shown graphically in figure 1, the periods required for 90 percentage absorption of the various compounds were as follows: *a*), desoxycorticosterone acetate, 64 days; *b*), estriol, 93 days; *c*), androsterone, 120 days; *d*), α-estradiol, 180 days; *e*), α-estradiol dipropionate, 220 days; *f*), α-estradiol benzoate, 415 days. For unknown reasons, the data for the estrone pellets were so scattered that an absorption curve could not be accurately established; a very rough estimate

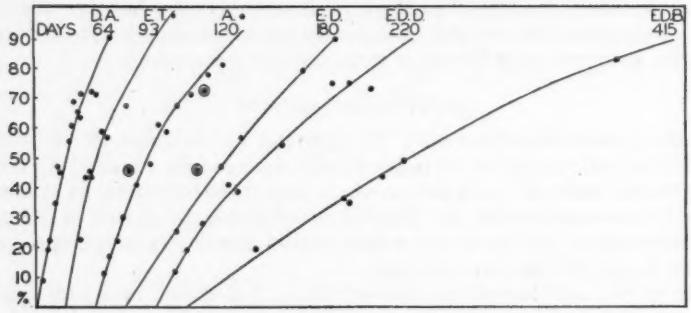

Fig. 1. Absorption rates and curves. The number of days required for 90 percentage absorption of desoxycorticosterone acetate (*D.A.*), estriol (*ET.*), androsterone (*A.*), α-estradiol (*ED.*), α-estradiol dipropionate (*ED.D.*), and α-estradiol benzoate (*ED.B.*) are indicated above. Each dot represents a pellet; the encircled dots indicate pellets having thick, a-typical capsules.

of the period required for 90 percentage absorption of the estrone pellets is 400 days.

None of the thirteen cholesterol pellets showed any appreciable absorption, even though seven of them had been left in situ for 140 to 143 days.

Study of factor of crystal size. The absorption rates of the pellets made of large and of small crystals of methyl testosterone, desoxycorticosterone acetate, and testosterone monopropionate are indicated in figure 2. There was no significant difference in the rate of absorption of pellets made of large crystals or of small crystals of a given compound. The relative periods required for 90 percentage absorption of methyl testosterone, desoxycorticosterone acetate, and testosterone monopropionate in the experiment with crystals of different sizes were 33, 48, and 55 days, respectively.

General observations. As in all previous experiments, no significant sex difference in the absorption rate of a given compound was detected (1).

Earlier observations on capsule formation were confirmed (1). A few cases in which thick, vascular capsules occurred and affected the absorption rate were again observed (fig. 1, 2).

Appreciable weight losses were observed at autopsy in 6 rats receiving pellets of estriol; 2 receiving androsterone; 2, α-estradiol; 6, α-estradiol dipropionate; 2, α-estradiol benzoate and 6, estrone.

DISCUSSION

A survey of the literature yields collateral information on the relative absorption rates in various animals, including man, of pellets of most of the compounds studied by the author. Estrone has been found to be absorbed at about one-half the rate of α-estradiol (2, 3, 4); α-estradiol is said to be absorbed from one and one-half to more than ten times as fast as its benzoate ester (5–8), but less rapidly than its dipropionate ester (8, 9). Estriol is variously reported to be absorbed less rapidly (4) and as rapidly (8) as α-estradiol. Finally, estrone is stated to be absorbed less rapidly than α-estradiol benzoate (10). The discrepancies may be partly due to the different types of animals used, but perhaps they are to a greater extent the result of the present incomplete understanding and evaluation of some of the factors governing the rates of absorption of pellets.

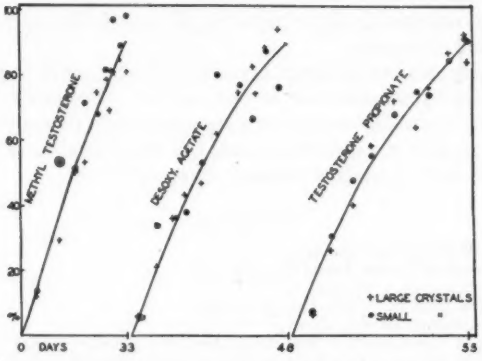

Fig. 2. Comparison of absorption rates of pellets made of large and small crystals. The number of days required for 90 percentage absorption of each compound is indicated below. Each dot or cross represents a pellet. The encircled dot designates a pellet having a thick, a-typical capsule.

The non-absorption of the cholesterol pellets was a surprising result, but is confirmed by a recent report that pellets made of a mixture of any one of several sex hormones with cholesterol were absorbed much more slowly than pellets consisting of the given sex hormone alone (11). The reductions in the absorption rates were proportional to the amounts of cholesterol included in the pellets.

In the experiment involving the use of pellets made of large and of small crystals, it was found that the relative periods required for 90 percentage absorption of methyl testosterone, desoxycorticosterone acetate, and testosterone propionate were 33, 48, and 55 days, respectively (fig. 2). In the first experiment reported in this paper, it was found that desoxycorticosterone acetate required 64 days for 90 percentage absorption (fig. 1). Both experiments were carried out under what are believed to have been essentially identical conditions and with a uniform technique, and the large discrepancy between 48 and 64 days cannot at present be explained. In an earlier publication it was reported that the 90 percentage absorption periods for methyl testosterone and testosterone monopropionate were 36 and 61 days, respectively; these values check reasonably well with the results for the corresponding

hormones in the present experiment (1). It is evident, however, that absorption rates cannot be reproduced perfectly until one or more as yet unrecognized variables are detected and brought under control.

The evidence from the second experiment indicates that, under the conditions of this experiment, crystal size is not a factor in the rate of absorption of a pellet. Presumably this is due to the construction and method of use of the author's apparatus, which crushes and compresses the crystals into a solid mass. Thus crystal size does not appear to be a significant variable affecting the rate of absorption of pellets made in this apparatus when the present technique is employed.

SUMMARY

A uniform technique was employed for the preparation, subcutaneous implantation in 87 sexually mature male and female rats, and removal of pellets of various crystalline compounds. The periods required for 90 percentage absorption of the pellets were as follows. Desoxycorticosterone acetate, 64 days; estriol, 93 days; androsterone, 120 days; α-estradiol, 180 days; α-estradiol dipropionate, 220 days; α-estradiol benzoate, 415 days. Cholesterol pellets were not absorbed. A loss of body weight sometimes occurred in the presence of all compounds except desoxycorticosterone acetate and cholesterol.

Pellets similarly made from large crystals and from small crystals of methyl testosterone, of desoxycorticosterone acetate, and of testosterone monopropionate were implanted in 59 sexually mature male and female rats. It was found that pellets made from large crystals of a given compound had been absorbed at the same rate as pellets made from small crystals of the same compound.

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FACTOR OF AGE IN THE RATE OF ABSORPTION OF, AND IN MAMMARY STIMULATION BY, TESTOSTERONE MONOPROPIONATE PELLETS IN RATS

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THE FOLLOWING EXPERIMENT was undertaken in order to investigate the possibility that the age of the host may be a factor in the rate of absorption of sex hormone pellets by rats. A frequently used androgen was selected as the test substance. Incidental gross autopsy observations were made on mammary gland stimulation.

METHOD

Pellets of testosterone monopropionate[1] were prepared by a standard technique so that they were subjected to uniform compression and were of uniform diameter; full details of the method have been described elsewhere (1). The pellets, weighing

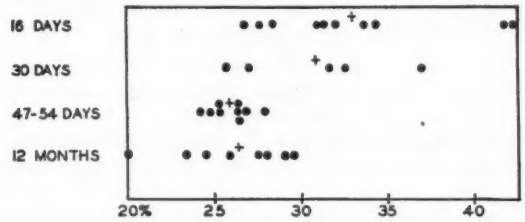

Fig. 1. ABSORPTION RATES AT DIFFERENT AGES. Each dot represents the percentage absorption of a pellet at the end of 12 days. The crosses indicate the average absorption of each age.

8.4 to 10.5 mg. each, were implanted in 33 rats of the following ages on the day of implantation: *a*), 4 male and 6 female littermates, aged 16 days ; *b*), 3 male and 2 female littermates, aged 30 days; *c*), 4 males and 5 females from two litters, aged 47 and 54 days; *d*), 5 males and 4 females, aged 12 months. Each rat received one pellet, implanted subcutaneously in the lower right abdominal quadrant under ether anesthesia. The pellets were carefully weighed before implantation. All rats were maintained under identical conditions.

Twelve days after implantation all pellets were removed surgically or at autopsy, dried to constant weight, and reweighed. The percentage loss in weight of each pellet was calculated and plotted graphically (fig. 1).

RESULTS

The relation of the age of the host to the absorption rate of the pellet is seen in figure 1. The average absorption rate for each group at the end of 12 days was as fol-

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[1] The testosterone monopropionate was supplied through the kindness of Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J.

lows: *a*), implantation in rats 16 days old, 33.0 per cent; *b*), implantation in rats 30 days old, 30.8 per cent; *c*), implantation in rats 47 and 54 days old, 25.9 per cent; *d*), implantation in rats 12 months old, 26.3 per cent. Since, as far as is known, the only essential variable factor was age, these results are directly comparable. It would thus appear that, during the first two months of life in the rat, there is a significant deceleration in the rate of absorption of testosterone monopropionate pellets. The difference between the absorption rates for the two older groups is not significant.

There was no significant sex difference in the absorption rates of the pellets.

The vaginae of the 6 females of the youngest group were open precociously at autopsy on the 28th day. In spite of the quantity of hormone absorbed, neither males nor females of this group showed any gross mammary development.

The 5 animals of the 30-day-old group were not autopsied.

Three males and 2 females of the 47 and 54-day-old group showed no appreciable gross mammary stimulation at autopsy on the 59th and 66th day. The mammary glands of one male and 3 females of this group, autopsied at the same time, had undergone slight stimulation.

The mammary glands of all 5 males and of 2 females of the 12-months-old group were moderately developed at autopsy, while the other 2 females showed marked mammary growth.

No microscopic studies were made of the mammary glands. On the basis of gross observations it appeared that, under the conditions of the experiment, testosterone monopropionate pellets can only evoke a rapid mammary gland development in rats aged more than two months.

DISCUSSION

The explanation for the decrease in the pellet absorption rate during the first two months of life is not at present apparent. However, it is evident that due allowance for such a decrease should be made in pellet studies with young rats. Ultimately, a similar decrease may be shown to occur in other young animals and with other types of pellets.

The estrogenic activity of testosterone and its esters is too well known to require comment; precocious opening of the vagina following administration of testosterone monopropionate is in line with earlier observations.

Astwood et al (2) and Reece and Mixner (3) have reported the histological details of stimulation of the mammary glands of immature and adult normal and castrate rats by the injection of testosterone and its esters. The data here presented seem to indicate that the administration of testosterone monopropionate for only 12 days does not cause macroscopic stimulation of the mammary glands of normal rats unless they are pubertal or postpubertal.

SUMMARY

Uniformly-made pellets of testosterone monopropionate, implanted subcutaneously in 33 male and female rats and recovered after 12 days, showed the following average absorption rates: implantation on the 16th day of life, 33.0 per cent; on the 30th day, 30.8 per cent; on the 47th or 54th day, 25.9 per cent; at the end of the 12th month, 26.3 per cent. The mammary glands of none of the prepubertal, some of the pubertal, and all of the postpubertal males and females showed grossly visible stimulation following treatment.

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OBSERVATIONS ON EFFECT OF ESTERIFIED ANDROGEN ON SEX EMINENCE OF THE CHICK

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THE PRACTICAL SIGNIFICANCE of the discovery by Fleishmann (1) of the existence of the anlage of the phallic knob in the domestic fowl (*Gallus gallus*) escaped the attention of science for the next twenty years. In 1925, Masui, Hashimoto and Ono (2) first announced their method of determination of sex in baby chicks partly based on size difference of the papillae in the two sexes. The method was introduced in a practical way on this continent in 1933 (3).

This rudimentary copulatory organ is present in all normal day-old and adult male chickens. Dove (4) reported the sterility of four yearling cockerels which showed no traces of the papillae. Goodale (5) found no papillae in capons. Masui corroborated this observation in 1927 (6). Domm (7) added his observation on this point, suggesting the existence of a hormonal control of this organ. The paucity of concrete information of the subject prompted the author to undertake an investigation of the effect of extraneous androgen on the development of the sex eminence in young chicks. The results of the investigation are reported in this paper.

MATERIAL AND METHODS

The study included four series of tests, two of them made simultaneously. Esterified male testicular hormone, testosterone propionate, served as the androgenic agent. Observations were made on chicks of both sexes.

In the first series, 20 cross-bred chicks of both sexes were used which were one day old at the commencement of the test. The cross involved was that of a Barred Plymouth Rock male with White Leghorn females. Half of the chicks were subjected to the hormone treatment, the remaining 10 serving as controls. The treatment consisted of the daily inunction of the papillae with a 3 per cent alcoholic solution containing 4 mg. of the hormone per cc. The control chicks were treated with the alcohol solution only. As it is difficult to measure accurately the amount of hormone applied percutaneously to the individual chicks, no attempt was made to determine the exact individual dose. However, the approximate dose was estimated by determining the amount of solution used during the 8-day period of treatment. In this way, it was estimated that each chick received, on the average, 4 mg. of the hormone in the course in the treatment. At the end of the 8-day period, the papillae of the chicks in both groups were examined and compared according to an arbitrary scale. The scale ranged from + to ++++, depending upon the size of the organ.

In the second series, run simultaneously with *Series I*, the hormone was injected into the pectoral muscle, sesame oil serving as the solvent. Twelve chicks, all males, were used in this test. Of these, 6 received the hormone, the remainder serving as controls. These chicks were of the same breeding as those used in *Series I*. The individual hormone dosage was 4 mg. in 0.5 cc. of oil, divided into 4 equal injections administered on 4 alternate days. Twenty-four hours after the fourth and final injection,

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TABLE 1. EFFECT OF PERCUTANEOUS APPLICATION OF TESTOSTERONE PROPIONATE ON THE PAPILLA OF THE CHICK

Hormone Treated Group			Control Group		
Number of chick	Sex	Size of papilla	Number chick	Sex	Size of papilla
1	♂	+++	11	♂	++
2	♂	+++	12	♂	++
3	♂	++++	13	♂	++
4	♂	+++	14	♂	+
5	♀	++	15	♂	++
6	♀	++	16	♂	++
7	♀	++	17	♀	+
8	♀	++	18	♀	+
9	♀	+	19	♀	+
10	♀	++	20	♀	+

when the chicks were 9 days old, the sex eminence was examined and its development compared in a manner similar to that adopted in *Series I*.

In *Series III*, observations were made on 100 Barred Rock male chicks. This number was divided into two equal lots by a method of restricted randomization based on the size of the papilla. Masui and Hashimoto (3) and Canfield (8) have shown that there is a wide range of variation in the size of the eminence in chicks of the same sex. It was thought advisable, therefore, to allot the chicks with papillae of various sizes equally to the treated and the control groups. The treated chicks received 3 injections of 2 mg. each of the hormone dissolved in 0.1 cc. of sesame oil, administered on alternate days. Thus the total hormone dose per chick was 6 mg. The control chicks received sesame oil only. As in *Series II*, the injections were made into the pectoral muscle, commencing when the chicks were 48 hours old.

In the final test, *Series IV*, observations were made on 91 Barred Rock pullet chicks, 45 of which received 6 mg. of testosterone propionate each, given in 3 equal injections on 3 alternate days. The remaining 46 served as controls.

RESULTS

Least definite results were obtained from *Series I* (percutaneous application). This was not surprising, because Zondek and Sulman (9) found that in rats the subcutaneous administration of hormone in oily solution is four times as efficient as the alcoholic percutaneous method. The same workers, however, concluded that in the chick the latter method of application is the more efficient of the two when comb growth is used as a criterion. It is possible, therefore, that the erectile tissue of the external genitalia of the rat or of the sex papilla of the chick is less responsive to the inunction method of hormone application than is the chick comb tissue. Nevertheless, it is apparent from the data in table 1 that there is a definite although not extensive

TABLE 2. EFFECT OF INTRAMUSCULAR INJECTION OF TESTOSTERONE PROPIONATE ON PAPILLA. MALE CHICKS

Hormone-Treated Group		Control Group	
Number of chick	Size of papilla	Number of chick	Size of papilla
21	++++	27	++
22	++++	28	+
23	++++	29	+
24	+++	30	++
25	++++	31	++
26	++++	32	+

stimulation of the papilla. It will be seen also that a slight increase in size of the papilla occurred in the few female chicks under the test.

The results were more clear cut in *Series II*, in which the route of administration was via the pectoral muscle. The papillae of the treated chicks showed more definite signs of enlargement than those in *Series I*. The data are summarized in table 2. This growth was paralleled by comb and wattle development (fig. 2, 3), a phenomenon which was completely absent in *Series I*.

An unexpected result developed in *Series II* in connection with growth of the head appendages. Frank and Klempner (10) and McCullagh and Guillet (11) concluded that it is necessary to isolate the treated from the control chicks to prevent contami-

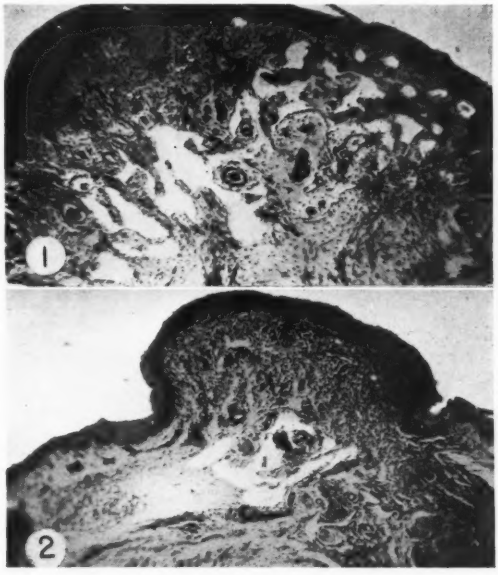

Fig. 1. TRANSVERSE SECTION OF SEX EMINENCE OF THE HORMONE-TREATED MALE CHICK. Notice cavernous spaces in the central zone. ×100. Fig. 2. Transverse section of sex eminence of the chick shown in fig. 4. Notice compactness of stroma of the eminence (compare with fig. 1). ×70.

nation of the combs of the latter with androgen from the birds treated by inunction. In the present work the results in *Series II* indicated the advisability of keeping the two groups separated, as precocious development of head parts was in evidence in both the treated and the control groups. The latter lot exhibited unmistakable signs of androgenic stimulation, such as the reddening and precocious growth of the comb and wattles (fig. 6). In addition there was a marked erythema of the eyelids. As there is no evidence of the existence of andromimetic qualities in sesame oil, it must be concluded that the androgenic-like stimulation in the control group was due to inunction of the head appendages by contact with the treated chicks. Since there is always a certain amount of seepage of oil from the injection site, it is easy to see how the chicks, which like to huddle under the heating pad, can contaminate each other with the hormone-impregnated oil. If one admits this possibility, then the head appendages would be the parts to show the effect of such stimulation.

It is apparent from table 2, however, that the size of the papillae was not affected

in these control chicks by the contact stimulation. The local stimulation of the comb under the conditions of this experiment did not affect the distal androgen-responsive tissues of the cloacal region. It is possible, of course, to envisage such peripheral stimulation if the quantity of the cutaneously applied hormone is great enough and the period of such contact application is spread over a long period of time.

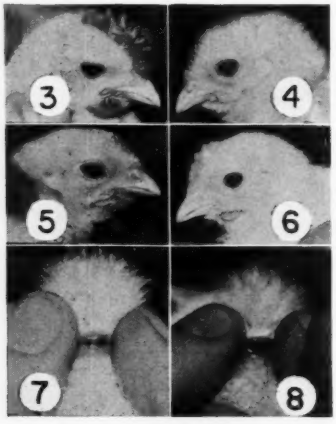

Fig. 3. HORMONE-TREATED MALE CHICK. Fig. 4. Control male chick injected with sesame oil only.
Fig. 5. MALE CHICK SHOWING NO SIGNS OF PRECOCIOUS COMB DEVELOPMENT. Sex eminence of this chick was treated percutaneously with hormone dissolved in alcohol. Fig. 6. Control male chick; comb and wattles show proliferation as a result of contamination through contact with the injected chicks.
Fig. 7. CLOACA OF THE MALE CHICK SHOWN IN FIGURE 3. The sex eminence is clearly seen on the lower lip of the cloaca. Fig. 8. Cloaca of the male chick shown in fig. 4. The sex eminence is not visible because of its small size.

The occurrence of this contact stimulation forced a slight alteration in the plan of conducting the tests in *Series III* and *IV*. Instead of keeping both the control and the hormone-treated groups together, as was done in the first two series, the two lots of chicks in *Series III* and *IV* were kept in separate compartments. To counteract any possible environmental differences between the compartments, the treated and the control groups were alternated between the compartments every 24 hours.

In *Series III* the treated groups showed early signs of precocious sexual development, in spite of the fact that Barred Rocks are a comparatively slow-maturing breed. Crowing was first noticed at 3 days of age, after only one injection of 2 mg. of the hormone. During frequent crowing spells as many as 15 of the 50 chicks in the treated lot could be seen crowing. Their behavior was similar to that described by Hamilton (12) for his 15-day-old Leghorn cockerels. The rudimentary copulatory organ gave signs of equally precocious development. On the fourth day after the commencement of the test, the papillae of the hormone-treated chicks appeared to be over twice as large as in the control group.

Both the crowing response and the growth of the organ were observed before there was any appreciable proliferation of the head appendages. The hormone-injected chicks also showed a marked reddening and 'puffing' of both the dorsal and the ventral lips of the cloaca. In appearance, this region closely resembled the description of cloacal hypertrophy observed by Wolff (13) in estrogen-injected chicks. The cloaca was soft and appeared tender to the touch.

The macroscopic appearance of the papillae in the two groups differed markedly.

In the controls the papillae appeared as small, translucent knobs; in the treated birds they were more opaque and whitish in color, resembling in this respect the papillae of adult males. An illustration of the organ of an injected chick is shown in figure 7 where it is plainly seen on the edge of the lower lip of the cloaca. No such structure is visible in the corresponding region of a control chick (fig. 8).

When the papillae of the injected chicks were examined under low-power magnification, the impression was received that the blood supply system of this organ is richly proliferated in comparison to that of the controls. Histological examination of the two organs (fig. 1, 2), corroborates this observation.

MacDonald and Taylor (14) studied the histology of the sex eminence in the domestic fowl. Their material ranged in age from four-day embryos to twelve-week-old chicks. They concluded that the papilla "possesses some histological and developmental characteristics of a true penis." Within its scope the present investigation supports this view. Besides being richly supplied with blood vessels, the central zone of the organ contains numerous cavernous spaces resembling those of the erectile type of tissue. During full erection these spaces are filled with blood. The spaces are particularly well defined in the papilla of a hormone-treated chick (fig. 1). The papilla of a control chick (fig. 2) has the appearance of a more compact mass of connective tissue.

The epithelial lining of the papilla, shown by MacDonald and Taylor to consist of both the horny outer epithelium and the inner columnar type, is considerably thickened in the organ of the treated chick. This explains the previously mentioned whitish appearance of the stimulated papilla.

At the same time, the horny layer of the latter appeared to be loose and in numerous sections of the organ from the treated chicks this layer is peeling from the inner, columnar cells. While the loose segment shown in figure 1 is undoubtedly an artifact, the appearance of such artifacts is so consistent as to suggest a general sloughing of the outer, cornified cells. This, of course, is not surprising in view of the rapidly proliferating stroma. None of the sections of the papillae from the control chicks show this condition. The presence of numerous pacinian corpuscles was noted, indicating the highly sensitive nature of the whole region. MacDonald and Taylor reported that the pacinian corpuscles rapidly increase in number with the growth of the chicken.

Comparison of the two sections in figures 1 and 2 shows, in cross section areas, the extent of the hypertrophy of the organ under the stimulation of the androgenic hormone. Obviously the total volumetric increase due to hormone action would be very much greater than is apparent from the examination of the sections. Measurements of the width of the base of the mounted organs (10 from each of the two groups) were secured by the use of camera lucida drawings, the values being expressed in arbitrary units. The papillae from the injected groups were approximately twice as wide as those of the control chicks. The difference was statistically significant at the 1 per cent point ($t = 9.66$).

The results in *Series IV* were, on the whole, similar to those secured in *Series III*. The papillae of the hormone-treated pullet chicks showed marked growth, on the whole, but not as great a development of the organ as was observed in the hormone-injected male chicks. The growth of the papillae preceded the visible signs of comb proliferation. Growing was observed in two chicks.

When the eminences of the chicks in both lots were graded according to the arbitrary scale and the values thus obtained were compared, the average size of the eminence of the treated chicks was more than twice that of the control birds. The difference was statistically significant at the 1 per cent point.

DISCUSSION

It is obvious from the observations presented that the so-called rudimentary copulatory organ of the domestic chicken is hormonally controlled. The fact that the growth of the papilla under the stimulus of the male sex hormone occurs in both sexes before there is any corresponding proliferation of the comb tissue might indicate a lower threshold value for the copulatory organ. However, it is possible that the apparent differences in the response thresholds may be due simply to the type of hormone used in this investigation. In that case the use of some other androgen would alter the situation. If this threshold difference is real, however, it is doubtful if it could be of any use in assay work because of the difficulty of measuring the organ. The volumetric method of Swinyard (15) could not be applied in this case because the papilla quickly collapses on isolation.

As already mentioned, it was not surprising that inunction of the cloacal region with the alcoholic hormone solution showed so little effect on comb proliferation (fig. 5) or on the growth of the papilla. This was paralleled by the absence of any signs of puffing of the dorsal and ventral lips of the cloaca, so much in evidence in the chicks intramuscularly injected with hormone. This may mean that the action of the male sex hormone on the cloacal tissue, including the erectile type of tissue of the papilla, is not entirely a direct one, but involves other members of the endocrine system.

Furthermore, the fact that Wolff (13) observed hypertrophy of the cloaca in male, female and 'intersex' chicks after injecting large doses of estradiol benzoate (unpublished results secured at this laboratory in general support Wolff's findings), indicates that the 'puffing' action is neither sex limited nor specific for the male sex hormone. It may be regarded, therefore, as a part of the syndrome of the action of large doses of extraneous sex hormones on a baby chick organism.

It is possible that the low order of response of the papilla to testosterone propionate in *Series I* (inunction method) was due to the relative impenetrability of its epithelium to the hormone. The thorough vascularization of the organ in that case would favor the action of the blood-borne hormone, leading to accelerated growth. In this regard the papilla differs from the chick comb which has been found to be very sensitive to the direct application of androgens (16).

SUMMARY

Testosterone propionate injected intramuscularly into baby chicks stimulates the growth of the sex eminence. This effect was observed in both sexes.

Testosterone propionate (Perandren) was supplied by the Ciba Pharmaceutical Products, Ltd., Montreal, Quebec.

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THE DISTRIBUTION OF FAT IN THE ENDOMETRIUM OF THE CASTRATE RABBIT AFTER TREATMENT WITH ESTRADIOL BENZOATE AND PROGESTERONE

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A SERIES OF EXPERIMENTS has been initiated in this department in order to ascertain the effects of estradiol benzoate and progesterone, not only on the uterus of the castrate rabbit but also on other organs and more especially on the endocrine glands. The results of the first study have already appeared (1).

The object of this paper is to examine the distribution of the lipoids in the endometrium of the rabbit during castration and under various experimental conditions in which estradiol benzoate and progesterone have been used, in order to discover whether the incidence and distribution of such lipoids as may be found depend on the presence or absence of these hormones.

Robson (2) has shown that estrogen antagonizes progestogen and unless proper combinations of these hormones are used, it is not possible to produce a progestational reaction. In these circumstances, the rabbit becomes a particularly useful animal since it is possible to examine the distribution of lipoids in the endometrium of the castrate rabbit when subjected either to estrogen or progestogen treatment alone or to such combinations of estrogen and progestogen as will lead either to the inhibition or to the stimulation of progestational responses.

MATERIAL AND METHODS

Rabbits weighing approximately 2 to 3 kg. were castrated. Twelve to 14 days after castration some of the animals were treated with small quantities of estradiol benzoate extending over a period of 7 days and then they received injections of estradiol benzoate alone, progesterone alone or combinations of both extending over a further period of 4 days. Twenty-four hours after the last injection the animals were killed by a blow on the neck, the uteri rapidly removed and fixed in formalin. Two to three days after fixation, sections of the material were cut on the freezing microtome, stained with Sharlach R for fat and counterstained with Ehrlich's hematoxylin. The sections were examined microscopically under oil immersion (×1200).

EXPERIMENTAL RESULTS

Rabbits 1, 2, 3; *table* 1. The uteri of *rabbits* 1 and 2 were examined 8 days after castration. Both uteri presented the same gross histological appearance with very few, small undilated glands in the form of epithelial folds dipping into the stroma. The stromal nuclei were either broad or oval, some being more pycnotic than the epithelial nuclei. The distribution of fat was variable. In *rabbit* 1, abundant fat globules of a coarse nature were scattered throughout the stroma (henceforth this fat will be referred to as 'stromal fat'), even extending into the circular and longitudinal

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muscle. Fat was present in groups of five to six globules both above and below the nucleus in the cells of the surface epithelium (henceforth referred to as 'epithelial fat'). In *rabbit* 2, the distribution of fat globules was considerably less, some cells containing no fat while others contained two to three globules. Fat globules, generally supra-nuclear in position, were also present in the cells of the epithelium lining the free surface of the uterus. The globules were coarse and only five to six globules were observed in an entire section.

The uterus of *rabbit* 3, examined 12 days after castration, also showed the presence of stromal fat in moderate quantities and very scanty fat restricted to a supra-nuclear position in the surface epithelium.

TABLE 1. EFFECT OF VARIOUS AMOUNTS OF ESTRADIOL BENZOATE, PROGESTERONE AND OF COMBINATIONS OF THE TWO ON THE DISTRIBUTION OF LIPOIDS IN THE UTERUS OF CASTRATED RABBITS

Rabbit Number	Pretreatment with Estradiol Over 7 Days	Estradiol Over 4 Days	Progesterone Over 4 Days	Progestational Reaction	Fat in Stroma	Fat in Glands	Fat in Surface Epithelium
	mg. total	*mg. total*	*mg. total*				
1	8-day castrate			−	++++	±	+++
2	8-day castrate			−	+++	−	±
3	12-day castrate			−	+++	−	±
4	0.07			−	+++	−	−
5	0.07			−	++	−	−
6	0.07	4		−	±	−	−
7			0.75	±	++	−	−
8			1.5	+	++	±	−
9			7.0	± to +	++	+	−
10			20.0	+	±	±	−
11	0.0058		0.75	++	+	−	−
12	0.0058		1.5	+++	+	±	−
13	0.0058	0.0025	0.75	+	±	−	−
14	0.0058	0.005	0.75	+	±	±	−
15	0.0058	0.01	0.75	+	++	±	−
16	0.0058	0.0025	1.5	+±	±	±	−
17	0.0058	0.005	1.5	++	++++	++++	−
18	0.0058	0.01	1.5	++	+++	+++	−
19	0.07	0.04	20.0	±	+++	−	±
20	0.07	0.4	20.0	−	++++	−	−
21	0.07	2.0	20.0	−	++++	−	+++
22	0.07	4.0	20.0	−	++++	−	++++

It follows that 8 to 12 days after castration the uterus shows the presence of stromal and epithelial fat in moderate amounts, the latter invariably being supra-nuclear in position.

Rabbits 4 and 5; table 1. The uteri of both of these rabbits were enlarged as compared with the castrate, and the glands, consisting of tube-like invaginations of the surface epithelium, were poorly developed. The cells of the stroma were fairly widely separated from each other, assuming a more compact appearance just below the surface epithelium.

In both cases, there was an absence of fat from the epithelium, while the distribution of stromal fat was variable. In *rabbit* 4, the majority of stromal cells contained one or more globules of fat while in *rabbit* 5, almost every cell contained two to three coarse globules of fat, this distribution being more marked in the edematous part of the stroma than in the compact region below the surface epithelium.

Thus, while a small quantity of estradiol benzoate does not cause any appreciable change in the distribution of stromal fat, it does cause the disappearance of all fat from the epithelium.

Rabbit 6; table 1. The histological appearance of the uterus of this animal was similar to that in *rabbit 4* and *rabbit 5* but the increased quantities of estradiol benzoate had caused marked changes in the distribution of the fat. Both in the surface and in the glandular epithelium, there was a complete absence of fat, while in the stroma fat globules were extremely rare, occurring as one globule in every fiftieth cell.

As compared with the castrate and with *rabbits 4* and *5*, which received relatively small quantities of estradiol benzoate, large quantities of estradiol benzoate apparently caused the complete disappearance of fat from both the surface and glandular epithelia and markedly reduced the stromal fat to almost negligible quantities.

Rabbits 7, 8, 9 and 10; table 1. In this experiment the rabbits received no estradiol benzoate but received progesterone alone from the outset. The uterus of *rabbit 10* (total dose, 20 mg. over a period of 4 days), showed a progestational response corresponding to a +1 reaction. The cells of the slightly dilated glands contained elongated, deeply staining nuclei, some of which were undergoing mitosis. As a result of the increase in the glandular activity, the stroma was considerably reduced. Fat globules were extremely scanty in the stroma and generally localized immediately below the surface epithelium. In the glandular epithelium, fat globules were correspondingly few and were observed in only four instances in an entire section. In every case, however, the globules were infra-nuclear in position.

In *rabbits 7, 8* and *9* stromal fat was present in greater abundance than in *rabbit 10*, one globule being found in every second to fourth cell, while the amount of epithelial fat was variable, though never present in abundance. *Rabbit 7* showed a complete absence of fat from the epithelium, *rabbit 8* showed only two infra-nuclear globules in the glandular epithelium in an entire field, while in *rabbit 9*, one globule appeared in at least one cell of almost every gland. The progestational response varied between ± and +1.

From these four rabbits, *7, 8, 9* and *10*, it follows that in comparison with the castrate and with rabbits treated with estradiol benzoate, treatment with progesterone alone in total amounts of 1.5 mg. and over not only causes the disappearance of the scanty supra-nuclear globules of epithelial fat, but also leads to the appearance of very occasional globules of fat, which are invariably infra-nuclear or basal in position, in the cells of the glandular epithelium. The amount of basal fat does not increase proportionately with the absolute amount of progesterone injected. The absence of epithelial fat after a total dosage of 0.75 mg. of progesterone in *rabbit 7*, was associated with a ± progestational response in the uterus. As all of the other rabbits showed a +1 response, it appears that the presence of basal epithelial fat is dependent at least upon a progestational reaction in the uterus even though it is not maximum. Total amounts of progesterone up to 7 mg. caused a decrease in the stromal fat as compared with the castrate and with *rabbits 4* and *5*, but large quantities of progesterone (20 mg.), caused an almost complete disappearance of stromal fat, similar to that produced by large quantities of estradiol benzoate in *rabbit 6*.

It has been shown that estradiol benzoate alone will not cause the appearance of basal epithelial fat but that progesterone alone in amounts of 1.5 mg. and over will produce a progestational response and the appearance of fat, however scanty, in the base of the epithelial cells. Neither the progestational response nor the amount of basal fat are enhanced by increasing the dosage of progesterone. It was therefore necessary to discover in what way these two reactions would be affected if animals received progesterone treatment after previous sensitization with estradiol benzoate.

Rabbits 11 and 12; table 1. After pre-treatment with estradiol benzoate (0.00084 mg. daily for 7 days), rabbits were given a total of 0.75 mg. and 1.5 mg. of pro-

gesterone, respectively, over a period of 4 days. In each case the uterus was enlarged and showed an increased progestational response as compared with *rabbits* 7, 8, 9 and 10. Nevertheless, despite the marked glandular proliferation in both cases, fat was absent from the epithelium in *rabbit* 11. It was present, however, in small quantities in not more than 8 to 10 cells in the entire section in the form of extremely fine globules, invariably infra-nuclear in position in *rabbit* 12. Fat was present in the stromal cells, generally one globule per cell.

This experiment established the fact that although small quantities of estradiol benzoate enhance the action of progesterone, the progestational response is not necessarily associated with increased epithelial fat. When amounts of less than 1.5 mg. of progesterone are used, fat is entirely absent from the epithelium (*rabbit* 11). Further, it may be concluded that while basal epithelial fat will not appear in the absence of a progestational response, nevertheless, it may be absent in the presence of a progestational response (*rabbit* 11).

The final experiment was planned to discover the effects of various combinations of estradiol benzoate and progesterone on the distribution of fat in the stroma and the glandular epithelium after the rabbits had received pre-treatment with estradiol benzoate. The experiment was divided into three series. In the first, 0.75 mg. of progesterone was used with various concentrations of estradiol benzoate (*rabbits* 13, 14, 15); in the second, 1.5 mg. of progesterone was combined with quantities of estradiol benzoate similar to those used in the first series (*rabbits* 16, 17, 18); in the third, 20 mg. of progesterone was combined with large quantities of estradiol benzoate (*rabbits* 19, 20, 21, 22).

Rabbits 13, 14, *and* 15; *table* 1. All of the uteri showed a varying degree of progestational response. The glands were long and narrow, with slightly dilated lumina, the nuclei of the cells were much elongated and crowded together. Occasional mitotic figures were present. In all cases, the stromal fat was much reduced as compared with the castrate and resembled the scanty distribution seen in *rabbit* 6. *Rabbit* 15 was an exception for this uterus showed as much stromal fat as the uteri of *rabbits* 7, 8 and 9. In all of the rabbits, epithelial fat was extremely scanty but, when it did occur, it was found in a supra-nuclear position.

Rabbits 16, 17, *and* 18. Again, varying degrees of progestational responses were elicited in these animals. The combination of estradiol benzoate and progesterone used in *rabbit* 17, produced the largest amount of fat yet observed in both the stroma and the epithelium, and its presence was associated with a +2 progestational response in the uterus. The lumina of the glands were very long and narrow and the epithelium was composed of tall cells with elongated nuclei occupying three-quarters of the cell. Fat globules were present in the epithelial cells in groups of as many as twelve fine droplets, lying most frequently in the infra-nuclear region of the cell. These fat globules were totally different from the coarse fat droplets seen chiefly in the supra-nuclear region of the epithelium of the castrate rabbit. In some glands fat was observed in every cell while in others there was none. Fat seemed to be absent most frequently in those parts of the glands which were situated in the deepest part of the endometrium lying near the inner layer of muscle. As a result of the increased glandular surface, the stroma was very much reduced but two or more globules of fat were present in every cell. Thus there was as much fat in the stroma of the uteri of *rabbits* 17 and 18 as in the castrate uterus. Fat was abundant in the glandular epithelium in *rabbits* 17 and 18 and was invariably basal in position whereas in *rabbit* 16 fat was extremely scanty, though basal in position.

Rabbits 19, 20, 21 *and* 22; *table* 1: With the exception of *rabbit* 19, all of the animals gave a negative progestational response. As seen from the table, a combination

of 4 mg. of estradiol benzoate and 20 mg. of progesterone was associated with as much stromal fat as in *rabbit 17* and was especially abundant in the sub-epithelial regions, parts of which were undergoing necrosis. The fat globules were irregular in size and generally of a coarse nature. The enlarged uterus was lined by a cuboidal epithelium which became flattened in parts. Glands were absent from the uterus. In some cases the epithelium itself seemed to have become involved in the necrotic processes by an extension from the underlying stroma. In the cells of the surface epithelium, two to eight fat globules were present in almost every cell and were distributed above and below the nucleus. The distribution of these fat-containing cells was patchy, for intervening groups of cells were found to contain no fat globules. The results in this series of experimental *rabbits 13* to *22* show that when small quantities of both estradiol benzoate and progesterone were used (*rabbits 13, 14, 15*), the amount of stromal fat is not obviously related proportionately to fluctuations in dosage of estradiol and progesterone. The same conclusion is arrived at when considering *rabbits 16, 17* and *18*. On the other hand, when large quantities of hormone are used, there is unquestionably an increase in the fat in the stroma which must be regarded as significant. In those uteri in which a progestational response was elicited the fat in the gland cells, with one exception, *rabbit 13*, was exclusively basal in position, although it was greatly increased in *rabbits 17* and *18* as compared with *rabbits 14, 15* and *16*.

DISCUSSION

The total lipoids in the endometrium of the pig and of the monkey, as determined by chemical methods, fluctuate during the menstrual cycle (3, 4). Okey, Bloor and Corner (3) drew attention to the increase in lecithin and free cholesterol in the uterus of the sow during the luteal phase of the cycle. Van Dyke and Chen (4) reported that in normal monkeys the total lipoids of the uterine and vaginal mucosae are highest during the secretory stage. Although these two investigations showed that an increase in the lipoids of the uterus and vagina in castrated monkeys after treatment with estrogen and progestogen could be produced, it was not identical with that obtained in the normal cycle.

The first serious attempt to examine histologically the lipoids in the endometrium during the menstrual cycle was made by Aschheim (5). He found fat in the uterine gland cells of 10 per cent of the specimens of human endometria in the first half and in 80 per cent of the specimens in the second half of the cycle. He concluded from this that fat found so frequently in the premenstrual condition was due to intense cellular activity in preparation for implantation. These observations of Aschheim as well as his interpretation of the presence of the fat, were seriously questioned by Froboese (6) who regarded this premenstrual increase of fat as evidence of a degenerative change preceding menstruation.

Gillman (7, 8) extended Aschheim's observations, and at the same time indicated that in normal women, during menstruation and pregnancy and in women subjected to experimental procedures, the distribution of fat in the gland cells was related in a causal manner to the optimum metabolism of progestogen and estrogen. In fact, the basal fat could be used as an indicator of optimum progesterone metabolism.

Bourg (9) noted an almost complete disappearance of fat granules from the cells of the uterine epithelium of the mouse during estrus, and a re-appearance in metestrus in the form of fine granules, principally in the base of the cell. He indicated that the uterine epithelium and, to a lesser extent, the uterine glands, showed these cyclical variations in the number and appearance of fat globules. He attempted to influence the distribution of fat experimentally by injections of a follicular substance. In the

castrate rat the epithelium was full of big fat granules, whereas after small quantities of estrogen there was a complete disappearance of this fat.

Bourg's findings in the rat were largely confirmed by Reese (10). Reese stated, however, that when the epithelium lining the uterine cavity was full of fat globules, the amount of fat in the gland cells was negligible, and that there was no evidence of cyclical change in the fat distribution in the glands.

From the experiments in rabbits it follows that after castration relatively large quantities of fat are present in the stroma, and scattered globules are found in the supra-nuclear region of the surface epithelium. The distribution of fat in the rabbit endometrium after castration is, in many respects, similar to that found in the castrate rat uterus, and is probably analogous to a similar condition existing in the endometrium of menopausal women.

Moreover, the distribution of the lipoid in the endometrium of the castrated rabbit can be profoundly altered by the administration of estradiol benzoate. It has been shown from the results in *rabbits* 4 and 5 that moderate quantities of estradiol benzoate resulted in the disappearance of what fat there was in the epithelium of the castrated rabbit, whereas the stromal fat was either unaffected or slightly decreased. On the other hand, 4 mg. of estradiol benzoate (*rabbit* 6) was associated with a complete absence of fat in the epithelial cells, and the presence of one small globule in every fiftieth stromal cell. Reese attributed the absence of osmiophilic granules from the epithelium of the rat during estrus to an altered metabolism. It follows then from the rabbit experiments above, that the altered metabolism leading to the disappearance of fat is provoked by estradiol benzoate which is more effective when given in large quantities.

While estradiol benzoate alone leads to a reduction of lipoids, not only as determined chemically (4) but also histologically, as compared with the castrate, the effects produced by progesterone alone administered to animals not previously sensitized with estradiol benzoate, depend largely on the dosage.

Small quantities of progesterone, up to 7 mg., produce a slight decrease in the fat of the castrate rabbit uterus, whereas after 20 mg. there is scarcely any stromal fat. In this respect progesterone behaves in a manner similar to that described for estradiol benzoate. In the epithelial cells, the effect of progesterone was the same as that after estradiol benzoate, with this exception, that although the fat was reduced after progesterone treatment, single globules were found in occasional gland cells, confined exclusively to the infra-nuclear region of the cell. This phenomenon is found neither in castrate animals nor in castrate animals treated with large or small doses of estradiol benzoate. It is, therefore, a reaction which can be regarded as dependent, in the first instance, exclusively on the presence of progesterone. The amount of this basal fat apparently is not related to the dosage as seen from *rabbits* 8, 9 and 10. There are definite limits within which the epithelial fat makes its appearance for in *rabbit* 7 0.75 mg. of progesterone did not produce any basal fat.

It is popularly thought that for optimum progesterone action it is necessary first to sensitize the uterus with estradiol benzoate. On this basis, *rabbits* 11 and 12 were given 0.07 mg. of estradiol benzoate over a period of 7 days, followed by a total of 0.75 mg. and 1.5 mg. of progesterone respectively over a 4-day period. In *rabbit* 11 the treatment evoked a +2 progestational response in the uterus, but there was no basal fat, and the stromal fat was reduced as compared with the castrate uterus. In this respect, it must be remembered that 0.75 mg. of progesterone administered to animals not previously sensitized with estradiol benzoate produced neither a progestational reaction nor basal fat. When the progesterone is increased to 1.5 mg.,

previous sensitization with estradiol benzoate enhances the progestational reaction and leads to the appearance of basal fat in occasional cells. From the results in *rabbits 11* and *12* which received a total of 0.75 mg. and 1.5 mg. of progesterone, respectively, after previous sensitization with estradiol benzoate, it seems that basal fat is not so much dependent upon the intensity of the progestational reaction, although this is an important factor, but rather on the amount of progesterone. Treatment with estradiol benzoate first, followed by progesterone, does not enhance the action of progesterone with respect to basal fat, but it does lead to an increased progestational reaction.

Thus the concept that estradiol benzoate acts first, and then progesterone, does not seem to be entirely true, for there is evidence to show that, by experimental procedures, it is not possible to obtain an optimum distribution of lipoids. The possibility of the effects of estradiol benzoate and progesterone in combination remained to be considered.

Black, Heyns and Gillman (11) have recently shown that the increased lipoid in the luteal phase of the human cycle not only is dependent upon the metabolism of progesterone alone, although this is essential, but also upon the metabolism of estradiol benzoate which plays an equally important part in maintaining the balance required for optimum luteal effect. In the rabbit, by using suitable combinations of estradiol benzoate and progesterone, it was possible to obtain a +2 progestational response in the endometrium, accompanied by the presence of numerous fine globules of fat in the infra-nuclear region of the glandular epithelial cells, and of large quantities of stromal fat, which equalled that described for the castrate. It must be noted, moreover, that basal fat when it does occur in the human endometrium is found in a very large proportion of glandular epithelial cells, whereas in the rabbit, even under optimum conditions, it occurs in considerably lesser quantities than in the human endometrium.

That there is a delicate balance between these two hormones has been shown by the fact that when the balance was upset, either by varying the quantity of estradiol benzoate with a constant amount of progesterone, or vice versa, it was still possible to obtain a progestational reaction, although its intensity was decreased and the amount of basal fat was immediately lowered. Van Dyke and Chen were unable to reproduce experimentally in castrates by injections of estradiol benzoate and progesterone the same distribution of fat as is found in the normal uterus at the height of luteal activity. It is possible that they failed because the balance of hormones injected in their experiments was not the optimal one that occurs in normal animals.

It appears then that only that combination of estradiol benzoate and progesterone which will give a plus 2 progestational reaction will be accompanied by an optimal distribution of fat in the glandular epithelial cells and in the stroma. On the other hand, certain combinations of estradiol benzoate and progesterone which are incapable of causing a progestational reaction lead instead to profound vascular changes (1), and the total amount of fat in histological section may appear to be the same as in the uterus after optimum treatment with these hormones. The combinations of hormones produce not only vascular reactions but also necrosis of the stroma and surface epithelium. The nature of the numerous coarse globules of fat found above and below the nucleus in the surface epithelium is in no way to be compared with the fine infra-nuclear globules found in the highly active endometrium of *rabbit 17*. In such instances, the abundant, large globules in the stroma associated chiefly with necrotic areas, is evidence that this fat is related to degenerative changes. In this respect, therefore, the nature and distribution of the fat associated with endometrial necrosis

in the rabbit is very similar to that described in the human endometrium during menstruation (7).

Similarly, the shift of fat from an infra-nuclear to a supra-nuclear position in the endometrium of the rat was attributed to degenerative processes associated with metestrus in the rat (9).

Thus fine globules of basal fat in the glandular epithelial cells of the rabbit, and the presence of stromal fat are invariably associated with a progestational response, which is dependent not only on pretreatment with estradiol benzoate but also on the optimum metabolism of estradiol benzoate and progesterone after such pre-treatment; whereas coarse globules occurring in large quantities in the stroma and epithelium indicate an inhibition of a progestational response and are related to an unfavorable metabolism of estradiol benzoate and progesterone.

From the foregoing, it follows that although basal fat in the uterine gland cell of the rabbit is dependent on the presence of progesterone, its optimum distribution is determined not only by satisfactory pre-treatment with estradiol benzoate, but also on a suitable combination of estradiol benzoate and progesterone after the estradiol benzoate pre-treatment.

SUMMARY

Castration in the rabbit is associated with the presence of small globules of fat in the stromal cells and a diffuse distribution of fat in the surface epithelium.

Estradiol benzoate alone in small quantities does not affect the distribution of fat in the stroma but causes the disappearance of fat globules from the surface epithelium. Estradiol benzoate alone in large quantities causes the fat to disappear from the surface epithelium and the fat in the stromal cells is considerably reduced.

Progesterone alone in quantities of 1.5 mg. to 7 mg., not preceded by treatment with estradiol benzoate, causes the appearance of extremely scanty basal fat in occasional gland cells. Large quantities of progesterone (20 mg.) do not enhance the distribution of basal fat but tend to reduce it. Progesterone alone in quantities of 1.5 mg. to 7 mg. results in a slight diminution in the amount of stromal fat while 20 mg. of progesterone causes the fat in the stromal cells to be considerably diminished.

In rabbits previously sensitized with estradiol benzoate, combinations of estradiol benzoate with adequate quantities of progesterone, which are associated with a +2 progestational response, cause the appearance of abundant fat in the infra-nuclear region of the glandular epithelial cells and in the stromal cells.

Combinations of estradiol benzoate and progesterone which are unable to produce progestational responses in the uterus, despite large quantities of progesterone, are associated with an abundance of fat in the supra-nuclear and infra-nuclear region of the surface epithelial cells and in the sub-epithelial stromal cells. The use of these combinations of estradiol benzoate and progesterone are at the same time associated with a marked vascularity of the uterus which may even culminate in necrosis of the endometrium.

Evidence is brought forward to show that basal epithelial fat in the uterus of the rabbit is actually related in some way to the metabolism of a combination of estradiol benzoate and progesterone, enhanced by the presence of estradiol benzoate. The appearance of large fat globules in the stromal cells of the sub-epithelial layers following the use of large quantities of estradiol benzoate and progesterone, is associated with necrosis of the stroma and the large fat droplets present in the cells may be regarded as a result of degenerative processes.

Basal epithelial fat is essentially related to progesterone which, when suitably com-

bined with small quantities of estradiol benzoate in an animal previously sensitized with estradiol benzoat , may cause a more widespread appearance of lipoid in the infra-nuclear region of the gland cells than occurs after progesterone alone.

I wish to express my thanks to Professor R. A. Dart for the facilities provided in his department and for his encouragement. My thanks are especially due to Dr. Joseph Gillman for advice and criticism given during the progress of this work.

Dr. G. Stragnell of the Schering Corporation, Bloomfield, N. J. has generously supplied the crystalline progesterone and estradiol benzoate used in this investigation.

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NEUROENDOCRINE MECHANISM OF COLOR CHANGE IN BUFO ARENARUM HENSEL

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THE SKIN OF AMPHIBIA becomes pale in the light and dark in its absence (1, 2). The color is also modified by the action of some endocrine glands (1–7) and of the nervous system (1, 2, 8, 9, 10). We have endeavored to determine the influence of the endocrine glands (hypophysis and adrenals) and of the nervous system (eyes, diencephalon, spinal cord and peripheral nerves) on the color changes produced by light stimuli in the toad *Bufo arenarum Hensel* which is extensively used for experimental work in this Institute.

METHODS

Toads, *Bufo arenarum Hensel* of both sexes weighing from 80 to 160 gm. were used. The changes in the pigmentary system were recorded according to the color observed directly and the aspect of the melanophores of the deep layers of the skin observed under a microscope with oblique illumination. The following indexes of pigmentation were used: *index 1*, a pale green color, concentrated pigment; *index 2*, a light green color, pigment slightly dispersed; *index 3*, green color, pigment moderately dispersed, *index 4*, dark green color, pigment widely dispersed; *index 5*, black color, pigment in reticulum.

Hypophysectomy was carried out according to the Giusti and Houssay (11) technique and the adrenals were destroyed by electrocoagulation. Posterior lobe extracts were prepared by treating dried bovine posterior lobes with NaOH 0.1N for 3 minutes at 100°C filtering and discarding the precipitate. The melanotropic hypophyseal hormone was titrated by injection into hypophysectomized toads weighing 100 to 120 gm. (12) and recording the color changes as previously described. It was found that the response of different toads belonging to the same group was practically the same.

The melanic-dispersing action of the blood was studied by establishing a vascular connection between the pale legs of hypophysectomized toads and the upper half of pale or dark normal toads. The technique was as follows. The hind legs of hypophysectomized toads were prepared according to the Lawen-Trendelenburg procedure by introducing glass cannulae into the aorta (*A*) and the abdominal vein (*V*). The animal used as perfuser was injected with heparin and the cannula *V* was introduced into the abdominal vein. The urostyle of the perfuser was then cut off, and the cannula (*A*) was introduced in the aorta near its origin thus excluding all of the vessels which have their origin in the descending aorta, especially those of the kidney and adrenals.

Destruction of the nervous system was carried out by cautery or pressure with forceps. The stimulations were made with a Harvard coil, silver electrodes separated 1 mm., 10 v. in the primary, frequency 80 to 1000 stimuli per minute.

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RESULTS

Action of light. Under thes timulus of light toads turn pale. In 120 minutes of illumination the color changes from extremely dark green (*index* 5) to pale green (*index* 1). Absolute darkness has the opposite effect, the toads turning completely dark in 60 minutes.

Action of endocrine glands. Toads hypophysectomized when pale never turn dark. Dark toads (*index* 5) hypophysectomized in darkness, except for a dim red light, turn

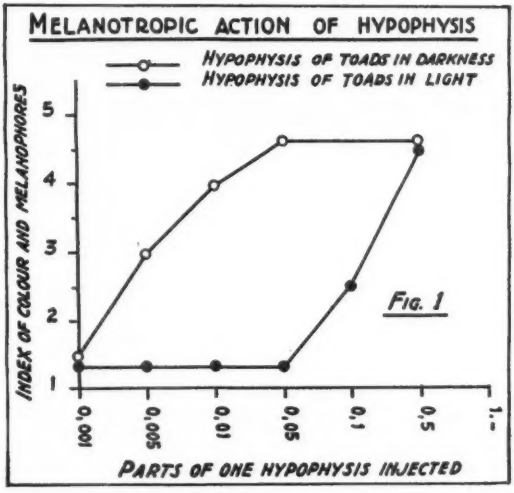

Fig. 1. Showing the melanotropic action of toad hypophysis.

pale in 60 minutes (*index* 1). The possible influence of adrenal secretion on the development of the pale color was excluded by the extirpation of the hypophysis in normal and adrenalectomized dark toads. All of these toads became paler, although the normal ones paled more rapidly than the adrenalectomized ones (60 and 120 minutes, respectively). The extirpation of the *pars principalis* did not modify the regulating action of the hypophysis on color.

The amount of melanotropic hormone in the hypophysis is diminished by the action of light and is increased in darkness (13, 14). The amount of hypophyseal tissue necessary to produce, in two hours, total darkening of hypophysectomized

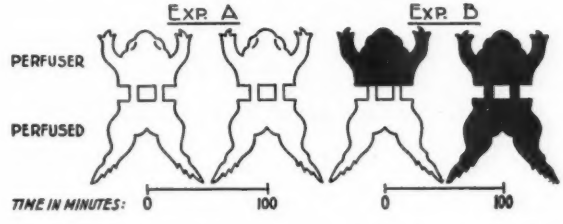

Fig. 2. Vascular anastomosis of pale hind legs to pale (*Exp. A*) and dark (*Exp. B*) toads.

toads weighing from 100 to 120 gm. was found to be 0.5 of an hypophysis of toads which had been exposed to light and 0.01 of an hypophysis of toads kept in darkness (fig. 1). The melanic-dispersing activity of blood varies with the light stimuli. If the hind legs of hypophysectomized toads are anastomosed with a pale normal toad,

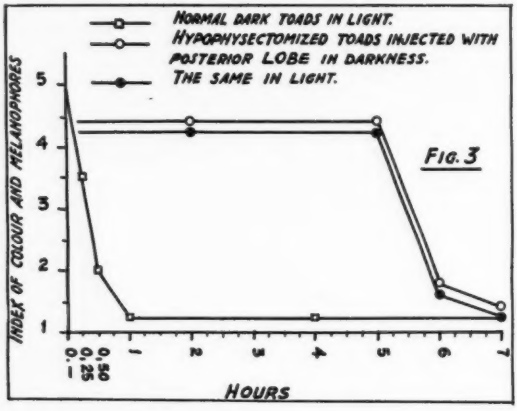

Fig. 3. Color index changes in toads under various conditions.

(*index* 1) they do not change in color (*Exp. A*, fig. 2). When the perfusing animal is dark (*index* 4), the perfused hind legs turn dark (*index* 4) in 60 minutes (*Exp. B*, fig. 2). This action of the blood can only be attributed to the presence of the melanotropic hormone, since according to the technique already described, the adrenals and the peripheral nervous system cannot act (fig. 2). Light did not modify the color

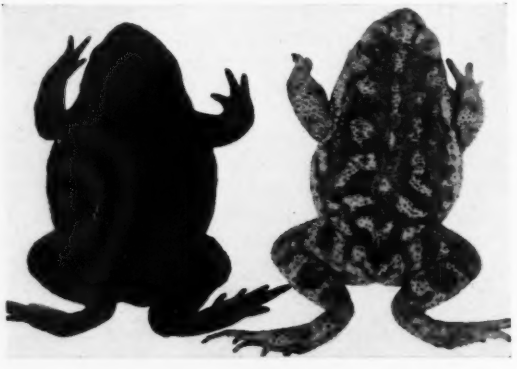

Fig. 4. Action of light on the color of normal (*right*) and *adrenalectomized* (*left*) *toads*.

of hypophysectomized toads darkened by injection of 0.1 cc. of posterior lobe extract. While normal dark toads (*index* 4) turn pale in 60 minutes (*index* 1), the injected hypophysectomized toads turned pale in 7 hours there being no differences between those kept in light and the controls kept in darkness (fig. 3).

After destruction of the adrenals the color of the skin darkened intensely. The illuminated adrenalectomized toads remained dark (*index* 4) while the normal control toads turned pale (*index* 1) in 60 minutes (fig. 4).

Adrenaline has an intense although brief effect on color. When 0.5 mg. of adrenaline is injected into dark toads (*index* 5) they turn pale in thirty minutes (*index* 1). After 180 minutes the toads recover their dark color. With 0.1 mg. of adrenaline there is some effect although it is less striking.

The antagonism between adrenaline and extract of the posterior lobe *in vivo* is weak. Hypophysectomized toads weighing 100 gm. received the necessary amount of extract of posterior lobe (0.1 cc.) for an appreciable darkening (*index* 4). Later they were injected with 1.5 mg. of adrenaline (*index* 5). While the normal control toads turned pale (*index* 1) the dark hypophysectomized toads changed only slightly (*index* 4).

Nervous system. The extirpation of the eyes or section of the optic nerves produces darkening of the skin and greatly reduces the pallor produced by light. These changes persist even three months after the operation. The destruction of the prequiasmatic zone or the *lobus infundibularis*, as well as the transection of the neuroaxis at the level of the diencephalon, produces darkening. The pallor-producing action of adrenaline is not modified. Stimulation of the *lobus infundibularis* in curarized toads (*index* 5) with a faradic current (90 stimuli per min.) produced total pallor in thirty minutes (*index* 1). Three hours after the stimulation the original color reappeared. Curarization or craneotomy have little influence on color (*index* 4). In a dark toad whose spinal cord or adrenals has been destroyed stimulation of the *lobus infundibularis* diminishes its paling effect (*index* 3). The excitation of the optic nerve has the same action as that of the *lobus infundibularis*.

Section of the sciatic or of the sympathetic nerves (cervical or lumbar) has scarcely any effect on the regulation of color, since the denervated areas respond like the normal ones to light and to pharmacologic stimuli (adrenaline, *F*933, potassium and nicotine), although the response is somewhat slower. Stimulation of intact cervical or lumbar sympathetic trunks scarcely pales the skin. (*Index* 2 or 3) which recovers the original dark color 15 minutes after the stimulus is over. The result is about the same in legs darkened by perfusion with extract of posterior lobe although there is a definitive paleness in them. Variations in the frequency of the stimulus (80–1000) have no apparent effect.

*F*933, a sympathycolitic, inhibits and cocaine increases the paleness produced by the excitation of the sciatic nerve.

DISCUSSION

Under the stimulus of light the skin of *Bufo arenarum Hensel* turns pale; in the absence of light the skin becomes dark. The conditions of darkness under which the toads were kept excludes all possibility of light stimuli to the hypophysis. This species, therefore, differs from others in which absolute darkness deprives the hypophysis of its normal stimulus, stops the secretion of the gland and the skin turns pale (15, 16).

The hypophysis is the factor that produces the darkening of the skin since its extirpation results in pallor in dark toads and prevents the pale ones from becoming dark. The light stimuli inhibit the melanotropic hypophyseal secretion, since *a*), the blood and hypophysis of pale toads contain less melanic-dispersing substance than those of dark ones; *b*) the color of hypophysectomized toads injected with small doses, of posterior lobe is not modified by light stimuli. The secretion of adrenaline is the factor which produces pallor, since destruction of the adrenals darkens the skin

permanently and adrenaline injections cause an intense paling of the color. The antagonistic action between hypophyseal and adrenal secretions does not seem to be on the melanophores since adrenaline scarcely pales hypophysectomized toads injected with posterior lobe extract. It is yet to be proved that adrenaline inhibits the secretion of melanotropic hypophyseal hormone in the toad as is the case in the rabbit (13).

The eye is the most important organ for perception of the light stimuli which affect the color. After its extirpation the skin darkens and the reflex responses to light are absent. This action is independent of traumatization of the stump of the optic nerve in the scar (16).

The infundibular centers stimulate the adrenal secretion and inhibit that of the hypophysis as can be demonstrated by *a*), destruction of infundibular centers after which the color darkens intensely and permanently; *b*), its stimulation which produces intense pallor; *c*), the fact that this last action diminishes, although it does not disappear, after the destruction of the spinal cord of the adrenals.

The peripheral nerves and the branches of the sympathetic nerves have a direct action upon the melanophores due to adrenergic fibres, as it is proved by the following facts. *a*), Its excitation has a paling effect on color either in isolated legs darkened by perfusion and in whole animals. *b*). The sympathycolitics (F_{933}) suppress the paling effect of the stimulation, and sympathicomimetics (cocaine) increase it. Their importance is negligible since areas of denervated skin respond as well as the normal ones to the various light and pharmacological stimuli.

SUMMARY

The rôle of endocrine glands and nervous system in the regulation of the color of *Bufo arenarum Hensel* has been studied. A technique for measuring the melanotropic action of blood is described. It was found that light produces pallor by ininhibiting the secretion of melanotropic hormone and by increasing that of the adrenals. The eyes are the most important receptor of the color regulating stimuli. The *lobus infundibularis* inhibits the secretion of melanotropic hormone and stimulates the secretion of adrenaline. Stimulation of the peripheral nerves concentrates the pigment of the melanophores but their importance is small in the regulation of color, humoral factors being predominant.

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AN EVALUATION OF VARIOUS METHODS FOR THE BIOASSAY OF DIHYDROTACHYSTEROL

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POTENT EXTRACTS of the parathyroid glands have been available for the treatment of clinical tetany for a number of years (1). Such extracts are usually standardized on the basis of their ability to elevate the serum calcium of normal dogs[1] (2). The maximal rise occurs from 9 to 18 hours after medication, usually nearer the latter.

Various activated sterols are also known to possess the power of elevating serum calcium. Among these are vitamins D_2 (crystalline calciferol) and D_3 (activated crystalline 7-dehydrocholesterol) and dihydrotachysterol,[2] designated in the literature as "A.T.10" (anti-tetanic preparation No. 10) (3, 4). The last named, although relatively devoid of antirachitic activity[3] is an effective hypercalcemic agent. An excellent review covering its discovery, and its chemical and physiological properties has been contributed by Holtz (5). Its use as a therapeutic agent was first reported in 1933 (6).

The present method for the standardization of dihydrotachysterol is that described by Holtz, Laquer, Kreitmair and Moll (7). This biological procedure, originally recommended for the assay of vitamin D, utilizes mice for the determination of the number of 'toxische Grenzdosen' or 'toxic borderline doses' per unit volume. By definition, one toxic borderline dose (T.B.D.) represents the least amount of dihydrotachysterol which, when fed daily over a 10-day period to each of a group of 8 to 10 male mice weighing initially 16 to 18 gm., will result, by the 11th day[4] in individual weight losses of at least 2.5 gm. or death in 50 per cent of the test animals. The commercial product is standardized to contain 200 such T.B.D. units per cc.

This assay technique for dihydrotachysterol, although based on weight losses or death of the test animals, may also serve indirectly as a measure of the hypercalcemic effects of the preparation, since anorexia, gastro-intestinal discomfort, diarrhea, and, in most species, nausea and vomiting, characterize a prolonged hypercalcemia. However, according to Reed, Struck and Steck (8) the toxic and hypercalcemic principles of vitamin D are not necessarily the same. They have observed death in dogs as a result of vitamin D intoxication, although these animals never showed any significant hypercalcemia. Conversely, they have observed a hypercalcemia of 24 mg. per cent and of 8 days duration in a human subject without any of the usual subjective symp-

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[1] The Collip extract (Parathormone) is standardized so that the unit is 1/100 of the amount required to raise the serum calcium of a normal 20-kg. dog by 5 mg. per cent.

[2] Sold by the Winthrop Chemical Company, Inc. under the trade name 'Hytakerol.'

[3] We have found that 1 mg. of crystalline dihydrotachysterol has an antirachitic effect equivalent to 80 U.S.P. units. Since of the 0.5 per cent of solids in the commercial material about 30 per cent are dihydrotachysterol, the antirachitic value for this preparation would be about 120 U. per cc. or 24 U. per mg. of solids. Harnapp (12) found this value to be 66 to 100 units per mg., Shohl and Farber (13) reported 100 U. per mg., and Correll and Wise (14) found 30 U. per mg. Our results therefore confirm those of Correll and Wise.

[4] If the animals are not medicated on the first day of the test and the intervening Sunday, the final observations are made on the thirteenth day.

toms or objective signs usually associated with serum calcium values of lesser magnitudes. It seems certain that these considerations apply with equal force to dihydrotachysterol since, as was pointed out by Werder (9), the hypercalcemic principle of activated sterols is not specific and is shared qualitatively by all activated (antirachitic) sterols.

The lack of specificity and the relatively poor quantitative reproducibility of assays of dihydrotachysterol by available methods suggested the need of a reliable assay technique based on the therapeutic indications for the product. The three new approaches to the problem which we have studied are *a*), determination of antirachitic vitamin D potency of unknown dihydrotachysterol preparations as against carefully

TABLE 1. ASSAY OF DIHYDROTACHYSTEROL PREPARATION A BY THE TOXIC BORDERLINE DOSE METHOD

Date of Test	Number of Animals	Dose, cmm.[1]	11-Day Observation: Animals Losing 2.5 Grams or Dying, per cent	18-Day Observation:[2] Animals Dying, per cent
5/1/41	9	7.5	77	33
	10	10.0	100	20
6/4/41	10	7.5	80	30
	10	15.0	90	90
7/16/41	10	7.5	60	30
	10	15.0	80	20

[1] Diluted with sesame oil so as to be contained in a volume of 0.2 cc.

[2] Eighteen-day observations are used as a further means of comparing the toxicity of preparations. Animals dead by the 11th day were included in this group.

assayed controls; *b*), determination of the hypercalcemic effects in rats; and *c*), determination of the hypercalcemic effects in dogs.

PROCEDURES AND RESULTS

Assay of dihydrotachysterol by the toxic borderline dose method. An evaluation of one specific preparation of dihydrotachysterol, designated hereafter as 'A,' based on assays on three different occasions by the toxic borderline dose method is shown in table 1.

The interpretation of these results would be that preparation A contains slightly more than one T.B.D. in 7.5 cmm., and for a final assay some further reduction in dose would be in order.[5]

The results obtained in this manner are, perhaps, as consistent as those of most bioassays of this type, nevertheless they leave something to be desired. For example, on the first assay a dose of 10 cmm. produced a greater response than a dose of 15 cmm. on subsequent tests. On two of the assays more animals died on the smaller dose than on the larger dose. It appears, therefore, that quantitative reproducibility of assay values by the T.B.D. method is difficult of achievement in spite of the fact that all factors within the control of the operators (age, sex, strain of animals, diet, temperature) are invariant. Since the stability of the preparations is not in question, the variability of the results may be largely attributed to such factors as differences in the degree of absorption of, or tolerance to, the preparation.

[5] In practice one seldom attempts to arrive at an exact numerical evaluation of a preparation by this procedure. Rather its effects are compared with those of a control preparation whose potency is known to be satisfactory, and the exact T.B.D. need not be determined.

Assay of dihydrotachysterol based on antirachitic potency. An assay of dihydrotachysterol based on antirachitic potency may be carried out in the following manner. Each cc. of commercial material (0.5% solution in oil) has an antirachitic effect at least equal to that of 110 U.S.P. units of vitamin D. If a certain preparation of dihydrotachysterol is taken as a standard, subsequent lots may be compared with it by determining their relative antirachitic effects. The data given in table 2 are illustrative.

From this test we may conclude that preparation *A* is slightly more potent than *B* and that both contain considerably more than the minimal required amount of the active principle. Repeated tests on the same material are consistent within the usual limits of vitamin D assay. While this procedure is promising as to reproducibility of results, it does not seem to be a logical means of estimating the hypercalcemic action of such materials which are, by reason of their origin, non-homogeneous. An evaluation method for dihydrotachysterol based on its antirachitic properties alone would

TABLE 2. RESULTS OF ASSAY OF DIHYDROTACHYSTEROL BASED ON ANTIRACHITIC POTENCY

Units of U.S.P. Reference Oil	Reference Oil	Average Degree of Recalcification (Standard U.S.P. line test)	
		Preparation A[1]	Preparation B[1]
0.5	+1.70		
1.0	+2.65	+2.97	+2.83
1.5	+2.92		

[1] Dose: 1/110 cc. per day for 10 days, diluted so as to be contained in 0.1 cc. Since 1939 the same total supplement has been administered in 6 doses.

be acceptable only if the potency ratio between the antirachitic and hypercalcemic factors were relatively constant for every preparation. Experimental evidence at hand, however, indicates that such a quantitative relationship may exist.

Assay of dihydrotachysterol based on hypercalcemic action in rats. Previous studies of the authors (10) suggested that standardization of dihydrotachysterol on the basis of its hypercalcemic effects in the albino rat might be feasible. Thus a unit oral dosage (0.5 cc. of a 0.5% solution in sesame oil) produced an average peak serum calcium value of 13.98 mg. per cent on the second day after medication. Two unit dosages (0.5 cc. of a 1% solution) under similar conditions gave a value of 15.16 mg. per cent. The marked differences between the responses to these two dosages at this interval (and at every other time interval on the curves) seemed to indicate that such a procedure merited further study.

In order to establish the normal relationship between dose and response, various amounts of the standard preparation were given as a single dose to groups of 15 to 25 albino rats weighing 175 to 225 gm. Usually a group consisted of an equal number of males and females although no constant sex difference has been apparent. The dose always was contained in 0.5 cc. of sesame oil, and the dihydrotachysterol concentration varied from 0.25 to 1.5 per cent.

The animals were fasted for 15 hours prior to medication, and were killed by decapitation 48 hours after medication. The blood was collected in centrifuge tubes, and calcium was determined on the separated serum by the Clark and Collip procedure (11). The serum from each animal was usually analyzed separately in order to calculate the standard deviation. If there is no interest in a statistical evaluation of the data, equally satisfactory results may be obtained by analyzing in duplicate pooled samples consisting of the same volume of serum from each animal.

The reasons for choosing the 48-hour interval are apparent from the previously published curves (10). Evidently analysis on the third or fourth day might have served just as well since after the peak values are passed the curves for the two dosages used remain essentially parallel. It is customary, however, in this type of assay to base conclusions on peak responses.

The results of this work are shown in figure 1. It appears that when the serum calcium values, determined as described above, are plotted against the logarithm of the dose, a straight line results. This method corresponds to plotting the increase in serum calcium against the logarithm dose since our untreated control value was

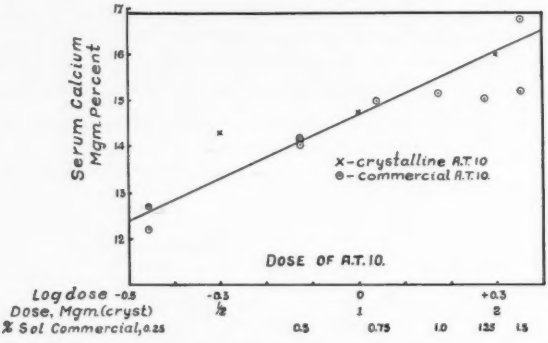

Fig. 1. Relation between serum calcium and dose of dihydrotachysterol in albino rats. Medication was given as a single dose in 0.5 cc. of oil; animals were killed 48 hours later.

established as 11.15 mg. per cent (10) and to subtract this value would merely shift the position of the ordinates. Those dosages which give serum calcium values of about 14 mg. per cent seem to be the most reproducible. For example, the exact values obtained after administering 0.5 cc. of a 0.5 per cent solution on three different occasions were 14.03, 14.11 and 14.14 mg. per cent. As previously mentioned, our preliminary study on a smaller group of animals had given the value 13.98 mg. per cent. This is a better agreement than one would expect since the calculated probable error of the mean at this dosage level for the larger groups of animals (15 to 25) is about 0.25 mg. per cent. The higher doses give more scattered values which usually, but not invariably, fall below the line. For assay purposes, it would, therefore, seem advisable to determine by preliminary test the amount of material which will give a serum calcium value of about 14 mg. per cent and to base the final evaluation on the result of administering this dose to a group of about 20 animals.

The difficulties encountered in obtaining satisfactory checks at the higher dosage levels may be due to progressively poorer correlation between dose and response as the maximal dose is approached. An analogy to this is the fact that U.S.P. assays for vitamins A, D, B, are more reliable when fractions of the curative dose are administered. It may also be that the higher doses are less completely absorbed. As for the possibilities of parenteral administration, intramuscular injections of dihydrotachysterol result in a very slow absorption, therefore are of no value in dealing with this situation. We have found it possible to administer intravenously as much as 50,000 units of crystalline vitamin D_2 in propylene glycol to rats. These animals show a greater degree of hypercalcemia than those receiving the same dose orally, and the differences between individuals are smaller. This technique, however, is not readily

applicable to dihydrotachysterol which as prepared commercially is non-crystalline and is dispensed in oil.

Based on the established curve, and scattering of points, it is possible to draw some conclusions as to the probable accuracy of assays of dihydrotachysterol carried out by this method. Taking the amount administered as a basis, the error for the amount found at the different points ranges from 0 to 43 per cent with an average of 15 per cent. The calculated error of the mean represented by the various points has been stated to be 0.25 mg. per cent (it is more at the higher dosage levels). The slope of the curve is such that a difference in serum calcium of 0.25 mg. per cent on the

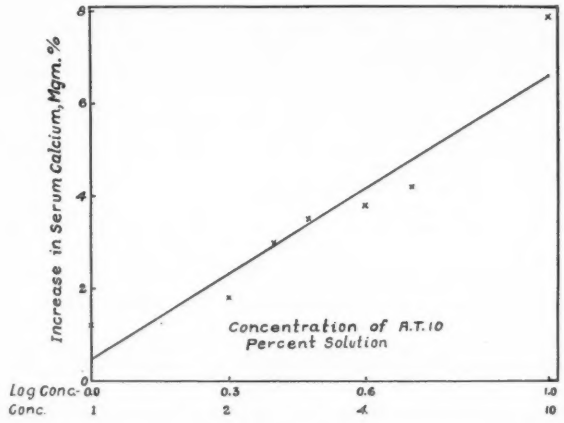

Fig. 2. Relation between the average increase in serum calcium and dose of dihydrotachysterol in dogs. Medication was 0.1 cc. per kg. (in oil) of the concentration indicated, as a single dose; blood samples were taken again 72 hours later and the increase calculated.

ordinate is equivalent to a difference of 0.18 mg. of dihydrotachysterol on the abscissa, therefore, at the 1-mg.-dose level one would expect a probable error of ± 18 per cent. This is a satisfactory agreement with the calculated average error.

Some comments on the work with crystalline dihydrotachysterol and the implications of the values found are necessary at this point. The commercial preparation is an oily solution of a resinous material which contains dihydrotachysterol. The exact proportion of the latter varies from one preparation to another. According to Werder about 40 per cent of the resin is dihydrotachysterol. Our test doses of the commercial material were selected on this basis to be equivalent to 0.5, 1.0, 1.5, 2.5, and 3.0 mg. of the active principle.

The hypercalcemic effects of crystalline dihydrotachysterol were tested at three levels in groups of ten rats. The values obtained are included in figure 1. It is evident that the proportion of the active principle in the commercial material must be less than 40 per cent. The points are plotted on the basis of 30 per cent purity and when this is done, two of the three values obtained for the crystalline material fall almost exactly on the curve. The limited amount of the crystalline material available did not permit a more exact evaluation, but in view of the position of the third point the purity value of 30 per cent may still be somewhat too high.

Correlation of data. In table 3 are shown data on the correlation of the three methods of assay thus far described.

Assay of dihydrotachysterol based on hypercalcemic effect in dogs. There were several reasons for the belief that the dog might be a very suitable animal for dihydrotachysterol assay. Since the use of the rat for assay purposes, according to the above procedure, necessitates the death of the animals, it seemed possible that dogs would be more suitable in that a group could be well-standardized as to diet, serum calcium values, and tolerance to the drug, and then serve repeatedly for purposes of assay. Finally, we hoped that dogs might react even more consistently to the drug than rats. For comparative purposes the technique used for bioassays of parathormone was

TABLE 3. CORRELATION OF ASSAYS OF DIHYDROTACHYSTEROL BASED ON TOXIC BORDERLINE DOSE, ANTIRACHITIC POTENCY AND HYPERCALCEMIC EFFECT IN RATS

Preparation	Toxic-Borderline-Dose Method	Antirachitic Potency,[1] Units	Hypercalcemic Effect, Equivalent to:
A	1 cc.=200 T.B.D.	1.5	0.50% solution
B	1 cc.=200 T.B.D.	1.35	0.40% solution
C	1 cc.=133 T.B.D.	1.0	0.49% solution
D	1 cc.=250 T.B.D.	1.75	1.03% solution
E	1 cc.= 80 T.B.D.	0.95	0.37% solution
F	1 cc.=100 T.B.D.	1.0	0.40% solution
Standard values	1 cc.=200 T.B.D.	1.0[2]	0.50% solution

[1] Dose of each: 1/110 cc. per day for 10 days, diluted so as to be contained in 0.1 cc. Since 1939 the same total supplement has been administered in 6 doses.

[2] The more satisfactory preparations usually assay better than 1 unit in 1/110 cc.

followed. Groups of five normal adult dogs averaging 10 to 12 kg. in weight were used for each assay.

Serum calcium was determined, and the animals were considered satisfactory if the value obtained was less than 12 mg. per cent (they were never less than 9.5, and usually were 10.6 to 11.2). The animals were kept on a calcium-rich diet (about 2 gm. per day) during the test period, and for several days before. The medication consisted of 0.1 cc. per kg. of the solution to be tested, which was always from 1 to 10 per cent concentration, inclusive, in sesame oil. As was shown by McChesney and Messer (4) the peak serum calcium value is found on the third day after medication. Blood samples were, therefore, taken at the 72nd hour post-medication and the increase in serum calcium was determined for each animal.

When the averages of such increases in serum calcium are plotted against the logarithm of the concentration of dihydrotachysterol, the points fall approximately on a straight line. It seems likely that as more animals were used the various points would tend to fall more nearly on the line. Evidence for this is the fact that those points which deviate most widely from the line represent the least number of animals (5 to 15) and those which fall most nearly on the line represent the larger number (up to 35).

The factor which mitigates against the practical use of this procedure is the extreme and unaccountable variations encountered between individuals. Occasionally some animals do not react to a medication at all, or at best only show an increase in serum calcium of 0.5 to 1.0 mg. per cent, while other animals on the same dosage may show an increase of 5 or 6 mg. per cent. It may be, as suggested by Rose, that the animals available to us were not sufficiently uniform, and were therefore unsuited to this sort of test. It is his belief that only about 20 of 100 dogs obtained in the laboratory are suitable for parathyroid assay. It should be mentioned that when we conducted a typical parathyroid assay with the groups of animals available to us, the

probable error of the mean of the increases in serum calcium was greater than when the same animals were treated with dihydrotachysterol. The average increase was slightly greater than that claimed for the product (parathormone).

Dogs are, of course, much less convenient animals to work with than rats, and an assay would hardly be practicable if it were necessary to use 25 or 30 dogs before one could with confidence decide on the correct result, or had to obtain 5 dogs for every one which was suitable for use.

SUMMARY

Various alternatives to the 'toxic borderline dose' procedure for the assay of A.T.10 have been studied. A determination of antirachitic potency of the preparation is satisfactory as a procedure but seems illogical on the ground that it is not necessarily a measure of hypercalcemic effect. A determination of hypercalcemic effect in rats seems to be a sound basis for assay, and the probable error of the method is of the order of 15 to 20 per cent. Application of the same principle to dogs has been only partially successful because of large individual variations in response.

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RÔLE OF NUTRITION IN RESPONSE OF BLOOD LIPIDS TO THYROIDECTOMY[1]

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IT IS NOW WELL ESTABLISHED that increased concentrations of lipids appear in the blood during thyroid deficiency (1, 2). In a recent communication from this laboratory, the blood lipids of dogs were observed for as long as 214 days after thyroidectomy during a nutritional state in which the same caloric intake was maintained during the entire period of observation, both before and after thyroidectomy (3). Changes in the levels of cholesterol esters were observed as early as 7 days after excision of the thyroid glands, and by the time 29 days had elapsed definite changes in total fatty acids had occurred. Not all lipid constituents of the blood responded to the same degree, for phospholipid and free cholesterol seldom showed pronounced increases after thyroidectomy. The increased concentrations of total fatty acids and cholesterol esters were not of constant occurrence after thyroidectomy. Rapidly fluctuating levels of esterified cholesterol and total fatty acids in blood were found in a number of the dogs after removal of the thyroid glands.

In the present investigation the relation of the nutritional state of the animal to these lipid responses in the thyroidectomized dog was investigated. It is shown that the rise in lipids of the blood that follows thyroidectomy in the dog can be completely *inhibited* by reduction in the caloric intake.

EXPERIMENTAL PROCEDURES

The excision of the thyroid glands was performed under ether anesthesia. In all cases one or two of the parathyroid glands were preserved intact with their blood supply. All other parathyroid tissue found was transplanted into neighboring muscle.

Before the operation all dogs were fed twice daily, at 8:00 A.M. and 4:00 P.M., a diet of lean meat and sucrose. The amounts fed to each dog are recorded in tables 1 to 4. In addition, each dog received daily throughout its period of observation 2 gm. of Cowgill's salt mixture (4), 4 gm. of bone ash, the vitamin B complex in the form of 5 cc. of a rice bran concentrate (Galen B, Type II liquid[3]), and vitamins A and D as contained in 3 cc. of sardilene oil.[4]

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[3] This concentrate was generously supplied by The Vitab Corporation, Emeryville, California.

[4] In each gram of this oil was contained a minimum of 100 A.O.A.C. chick units of vitamin D and 600 U.S.P. units of vitamin A.

TABLE 1. INFLUENCE OF FASTING AND REFEEDING UPON WHOLE BLOOD LIPIDS OF THE THYROIDECTOMIZED DOG T2

(All lipid values are expressed as mg. per 100 cc. of whole blood)

Weight	Period of Observation				Cholesterol			Total Fatty Acids	Phospholipids	Total Lipids
	After thyroidectomy	Diet per day								
		Days on diet	Meat	Sucrose	Total	Free	Ester			
kg.	days		gm.	gm.						
8.3	(11) Before	7	250	50	181	133	48	464	336	645
7.9	(0) Before	18	250	50	183	121	62	450	304	633
9.0	24	42	250	50	207	122	85	560	389	767
9.3	39	57	250	50	193	116	77	464	370	657
10.0	60	78	250	50	206	115	91	450	381	656
9.9	65	83	250	50	247	125	122	512	432	759
9.8	66	1	0	0	209	109	100	394	425	603
9.3	70	5	0	0	198	127	71	334	406	532
9.7	77	7	250	50	252	119	133	536	427	788
10.0	81	11	250	50	208	114	94	450	398	658
9.4	86	5	0	0	159	95	64	423	269	582
9.8	93	7	250	50	264	128	136	557	448	821
9.7	95	9	250	50	256	106	150	545	508	801
9.0	100	5	0	0	145	82	63	394	369	539
10.0	107	7	250	122	373	146	227	688	477	1061
10.0	112	12	250	122	220	118	102	463	362	683
9.4	117	5	0	0	158	86	72	450	364	608
9.0	176	8	37	7	197	141	56	412	372	609
9.0	182	14	37	7	166	89	77	478	398	644
8.5	193	25	37	7	181	107	74	424	370	605
8.1	205	37	37	7	170	89	81	478	366	648
7.7	219	51	37	7	188	109	79	435	404	623
7.2	231	63	37	7	185	107	78	440	441	625
7.1	233	65	37	7	175	105	70	427	357	602
7.0	236	68	37	7	165	110	55	430	424	595
7.8	249	13	250	50	278	136	142	549	446	827
7.9	255	19	250	50	271	146	125	555	527	826
7.6	260	5	0	0	106	104	2	424	379	530
8.1	273	8	250	50	325	142	183	580	516	905
7.8	278	5	0	0	172	95	77	418	385	590
7.6	390	9	250	50	326	136	190	806	470	1132
7.0	395	5	0	0	110	63	47	244	241	354

After thyroidectomy the dogs were subjected to alternate periods of fastingand refeeding. For periods of 5 to 7 days they were fasted and then refed for 7 to 68 days. At various intervals whole blood was removed for lipid analysis. The oxidative methods employed in the determination of blood lipids have been described elsewhere (5). Free cholesterol was determined in an acetone solution from which phospholipids had been precipitated.

RESULTS

Thirteen completely thyroidectomized dogs were studied in the present investigation. The results obtained in 6 of these are recorded here. In 2 dogs (tables 1 and 3) the period of observation after thyroidectomy extended for over one year (395 days). The shortest period of observation after thyroidectomy was 193 days (table 4).

Dogs T2, T3 and T5

Feeding at preoperative levels. Dogs T2, T3 and *T5* received identical treatment throught the first 233 days of their periods of observation (tables 1–3). Blood lipid measurements were made on two occasions before thyroidectomy, 11 days before and

again on the day of operation. The two preoperative values obtained for cholesterol, phospholipid and total fatty acids for each dog were in fairly close agreement. During the first 65 days after thyroidectomy these 3 dogs were fed the same diet that they had received before. On the 65th day after thyroidectomy, the concentrations of all lipid constituents except free cholesterol were far in excess of those found during their preoperative or control periods. The increases in esterified cholesterol were particularly striking: in *dog T2* it doubled and in *T5* it rose from a preoperative level of 22–29 to 100 mg.

Alternate fasting and feeding. During the next 52 days (shown in tables 1, 2 and 3, from 66 to 117 days after thyroidectomy) *dogs T2, T3* and *T5* were subjected to al-

TABLE 2. INFLUENCE OF FASTING AND REFEEDING UPON WHOLE BLOOD LIPIDS OF THYROIDECTOMIZED DOG T3
(All lipid values expressed as mg. per 100 cc. of whole blood)

Weight	Period of Observation				Cholesterol			Total Fatty Acids	Phospholipids	Total Lipids
	After thyroidectomy	Diet per day								
		Days on diet	Meat	Sucrose	Total	Free	Ester			
kg.	days		gm.	gm.						
10.5	(11) Before	7	316	63	161	106	55	406	318	567
10.7	(0) Before	18	316	63	176	109	67	416	306	672
12.4	24	42	316	63	236	115	121	570	466	806
12.7	31	49	316	63	325	145	180	538	515	863
13.0	39	57	316	63	263	126	137	563	488	826
13.4	60	78	316	63	284	140	144	416	437	700
13.4	65	83	316	63	257	138	119	488	497	745
13.4	66	1	0	0	274	135	139	410	457	684
12.6	70	5	0	0	199	114	85	442	416	641
13.0	77	7	316	63	320	155	165	567	468	887
13.3	81	11	316	63	304	135	169	618	453	922
12.7	86	5	0	0	159	98	61	423	336	582
13.1	93	7	316	63	311	136	175	643	437	954
13.0	95	9	316	63	322	145	177	662	570	984
12.4	100	5	0	0	178	100	78	445	406	623
13.5	107	7	316	135	368	148	220	764	558	1132
13.5	112	12	316	135	354	164	190	612	486	966
12.9	117	5	0	0	197	120	77	437	376	634
12.7	176	8	47	9.5	206	137	69	404	372	610
12.4	182	14	47	9.5	182	108	74	460	394	642
12.1	193	25	47	9.5	191	108	83	416	409	607
11.7	205	37	47	9.5	213	138	75	478	388	691
11.4	219	51	47	9.5	196	114	82	468	411	664
10.9	231	63	47	9.5	184	118	66	457	367	641
10.7	233	65	47	9.5	191	112	79	423	417	614

ternate fasting and feeding. Fasting for 5 days (from the 65th to 70th day of observation) produced falls in the levels of all lipid constituents. Thus in *dog T2*, total cholesterol fell from 247 to 198 mg. and the esterified portion from 122 to 71. The response of total fatty acids was more pronounced than that of phospholipids; the former fell from 512 to 334 and the latter from 432 to 406 mg. Essentially the same picture was observed in *T3* and *T5*, except that in these dogs the loss of total fatty acids was not so great as in *dog T2*.

For the next 11 days (from the 70th to 81st day of observation) these 3 dogs were fed the standard diet. In 7 days of refeeding, total cholesterol in the blood of *T3* reached the high value of 320 mg., an increase of 121 mg. above the preceding fasting level. Most of this rise was due to esterified cholesterol. Again, total fatty acid rose

decidedly in contrast to phospholipid, which showed but little change during this phase of altered nutrition. In $T2$, refeeding for 7 days failed to change the level of phospholipid observed on the 70th day of observation, at which time this dog had completed a 5-day fast.

From the 81st to 86th day of observation these 3 dogs were again deprived of all caloric intake with the exception of the small amounts included in the vitamin con-

TABLE 3. INFLUENCE OF FASTING AND REFEEDING UPON WHOLE BLOOD LIPIDS OF THYROIDECTOMIZED DOG T5
(*All lipid values expressed as mg. per 100 cc. of whole blood*)

Weight	Period of Observation				Cholesterol			Total Fatty Acids	Phospholipids	Total Lipids
	After thyroidectomy	Diet per day								
		Days on diet	Meat	Sucrose	Total	Free	Ester			
kg.	days		gm.	gm.						
9.6	(11) Before	7	285	56	132	110	22	370	382	502
9.9	(0) Before	18	285	56	142	113	29	352	360	494
11.0	24	42	285	56	244	127	117	480	400	724
12.1	39	57	285	56	268	150	118	555	436	823
12.7	53	71	285	56	228	130	98	525	467	753
12.6	60	78	285	56	223	127	96	475	387	698
12.5	65	83	285	56	220	120	100	462	430	682
12.6	66	1	0	0	203	116	87	406	406	609
11.8	70	5	0	0	169	114	55	415	390	584
12.2	77	7	285	56	232	115	117	521	449	753
12.5	81	11	285	56	232	145	87	507	439	739
11.7	86	5	0	0	159	107	52	350	315	509
11.8	88	2	285	56	157	101	56	386	410	543
12.5	93	7	285	56	215	123	92	551	492	766
12.3	95	9	285	56	249	117	132	492	508	741
11.7	100	5	0	0	169	102	67	380	406	549
12.5	107	7	285	128	274	146	128	552	444	826
12.5	112	12	285	128	273	139	134	607	461	880
11.7	117	5	0	0	187	115	72	427	420	614
11.5	176	8	43	8.5	161	105	56	435	376	596
11.7	182	14	43	8.5	174	116	58	444	382	618
10.7	193	25	43	8.5	208	138	70	350	428	558
10.2	205	37	43	8.5	141	107	34	450	378	591
9.9	219	51	43	8.5	189	102	87	368	347	557
9.2	231	63	43	8.5	170	107	63	404	398	574
9.5	233	65	43	8.5	178	107	71	360	352	538
9.0	236	68	43	8.5	172	111	61	458	484	630
9.7	249	13	285	8.5	250	132	118	515	492	765
9.7	260	5	0	0	175	102	73	361	392	536
10.4	273	8	285	56	303	116	187	600	478	903
9.9	278	5	0	0	214	114	100	402	368	616
10.0	390	9	285	56	318	155	163	862	556	1180
9.5	395	5	0	0	213	110	103	380	362	593

centrates. In $T3$ both the free and esterified cholesterol fell decidedly, while the total fatty acid fell from 618 to 423 and phospholipid from 453 to 336. In $T2$ the fall in phospholipid was more pronounced than in total fatty acid. In $T5$ significant decreases occurred in both total fatty acids and phospholipid.

During the next 9 days (from 86th to 95th day of observation) feeding of the preoperative diet was resumed. All lipid constituents in $T5$ showed decided increases in their concentrations in the blood. At the end of the first 7 days of refeeding the esterified cholesterol of $T3$ rose to 175 mg., an increase of 114 mg. The increases in the concentrations of total fatty acid and phospholipid in the blood of this animal were equally striking.

Between the 95th and 117th day of observation, two fasting periods and a single period of feeding were instituted. The most striking response of cholesterol to the ingestion of the diet is shown in the case of *T3*. In this animal total cholesterol rose to 368 mg. in the short period of 7 days after the preceding fast; the absolute increase in this short space of time was 190 mg. per 100 cc. of whole blood. Most of this rise was due to esterified cholesterol, which rose from 78 to 220 mg. The response of total

TABLE 4. INFLUENCE OF FASTING AND REFEEDING UPON WHOLE BLOOD LIPIDS OF THYROIDECTOMIZED DOGS T12, T13 AND T14

(All lipid values expressed as mg. per 100 cc. of whole blood)

Dog	Weight	Period of Observation				Cholesterol			Total Fatty Acids	Phospholipids	Total Lipids
		After thyroidectomy	Diet per day: Days on diet	Diet per day: Meat	Diet per day: Sucrose	Total	Free	Ester			
	kg.	days		gm.	gm.						
T12	9.6	(20) Before	42	300	10	168	112	56	314	362	482
	9.2	(16) Before	46	300	10	177	119	58	349	322	526
	11.5	40	86	300	10	270	128	142	510	468	780
	10.9	45	5	0	0	163	107	56	350	367	513
	10.6	186	7	300	10	284	129	155	562	411	846
	10.0	193	7	0	0	160	103	57	418	356	578
T13	10.4	(20) Before	42	350	10	159	113	46	402	447	561
	10.5	(16) Before	46	350	10	173	111	62	406	405	579
	13.3	40	86	350	10	322	143	179	566	503	888
	12.6	45	5	0	0	203	116	87	390	416	593
	11.2	186	7	350	10	256	130	126	555	467	811
	10.4	193	7	0	0	150	102	48	404	364	554
T14	12.4	(20) Before	16	410	10	189	123	66	432	432	621
	13.0	(6) Before	30	410	10	164	110	54	433	404	597
	18.2	40	70	410	10	270	124	146	513	446	783
	15.7	45	5	0	0	189	108	81	408	386	597
	12.9	186	7	410	10	200	95	105	450	388	650
	12.0	193	7	0	0	132	79	53	382	330	514

fatty acid and phospholipid during this same time was also marked; the former rose from 445 to 764 mg. in 7 days of refeeding, the latter from 406 to 558 mg. On the 7th day of refeeding total lipid attained the value of 1132 mg.

Chronic undernutrition. For the next 65 to 68 days the effects of chronic undernutrition were investigated. During this time each dog received 15 per cent of its original diet. Tables 1 to 3 show that on a diminished diet there occurred a steady loss in weight, and in *dog T2* the final weight at the end of the 68th day was about 1 kg. below its weight at the beginning of the experiment.

Under the influence of a markedly diminished daily caloric intake the levels of blood lipids in *dogs T2*, *T3* and *T5* failed to rise to the high levels previously noted during periods of regular feeding. In each dog, the feeding of the low-caloric diet was begun at the end of a 5-day period of fasting (see 117th day of observation after thyroidectomy), i.e. at a time when the concentrations of blood lipids had been reduced. In *T2* the levels of cholesterol found at the end of the 5-day fast were 158 mg. for total and 62 mg. for esterified; during the next 68 days the levels of each of these lipid constituents did not rise above the latter values. The same was true for total fatty acid. The fasting level was 450 mg., and the highest value observed throughout

the period of chronic undernutrition was 478 mg. Essentially the same thing was found in *dog T3* during its period of chronic undernutrition. These effects are reflected in the levels of total lipid. The fasting values (see 117th day of observation after thyroidectomy) for total lipid were 608, 634 and 614 in *dogs T2, T3* and *T5*, respectively; the highest values observed during the subsequent period of underfeeding were 648, 691 and 630 mg.

Alternate feeding and fasting. In *dogs T2* and *T5*, the period of chronic undernutrition was followed by a period (from the 236th to 395th day of observation after thyroidectomy) during which feeding of a normal diet and fasting were alternated. This was instituted in order to determine whether the levels of blood lipids at the

TABLE 5

		Cholesterol		Fatty Acids	Phospholipid
		Ester	Free		
Fasting	*t* value	4.33	2.03	2.24	2.58
	Probability due to chance	0.06%	6.6%	4%	2%
Refeeding	*t* value	3.22	1.76	2.34	0.74
	Probability due to chance	0.6%	10%	3.8%	46%

late intervals after thyroidectomy were as responsive to alterations in nutritional state as they had been earlier. The results recorded in tables 1 and 3 show that as late as 395 days after thyroidectomy nutritional state is still an important factor in determining the concentration of lipids in the blood.

Dogs T12, T13 and T14

Despite the fact that the effects of alteration in nutritional states, i.e. fasting and refeeding, were observed on 7 occasions in the 3 dogs shown in tables 1 to 3, nevertheless the spontaneous occurrence of fluctuations previously observed in the lipid levels of the blood of thyroidectomized dogs maintained on a constant caloric intake (3) made desirable a further extension of the present study. The fall in blood lipids with fasting and the rise observed with refeeding have been noted in 10 dogs in addition to those recorded in tables 1 to 3. The results shown in table 4 are typical of those observed in the 10 additional dogs. Forty days after thyroidectomy the blood lipids of *T12, T13* and *T14* were elevated considerably above their respective control or preoperative levels. Alternate fasting and feeding was then instituted. A fall in response to the former and a rise in response to the latter were again observed in the lipid levels of the blood. Thus the changes obtained here by altering the nutritional state of 13 dogs leave no doubt that they are a true response to nutrition and not spontaneous fluctuations such as previously had been observed in thyroidectomized dogs maintained on a constant diet.

DISCUSSION

Previous communications from this laboratory have shown that the lipid levels in whole blood of normal dogs remain fairly constant during changes in nutritional states (6, 7). Fasting in the normal dog for as long as 30 days produced only slight decreases in whole blood lipids (6). Moreover, the production of obesity, in which dogs were made to increase their weights by as much as 80 per cent, led to little or no rise in

total cholesterol of the whole blood, although a tendency for total fatty acids and phospholipids to rise was observed (7). Fasting apparently had a more pronounced effect upon the blood lipid level of the obese than of the normal dog.

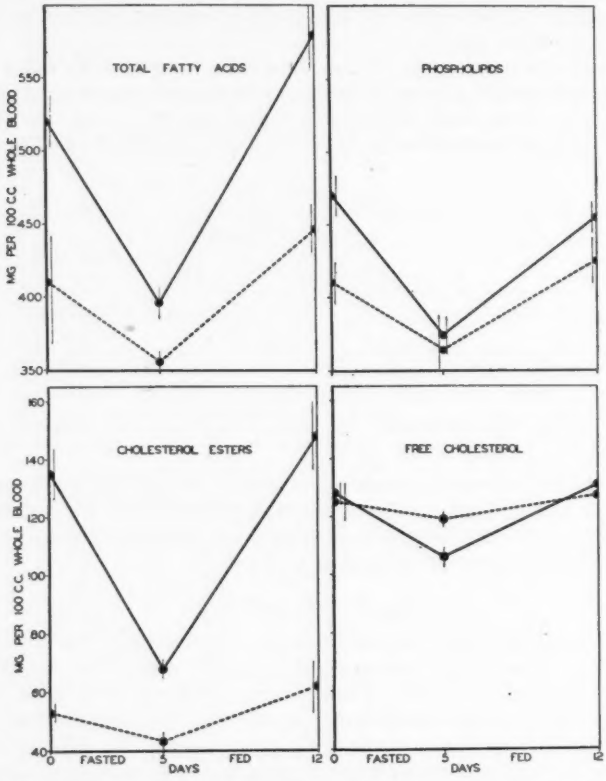

Fig. 1. Comparison of the normal and thyroidectomized dog in the changes produced upon whole blood lipids by fasting and refeeding.

Normal values were obtained from 5 normal dogs that were subjected to a 5-day fast which was followed by a 7-day period of refeeding. The thyroidectomized values were obtained from 6 dogs recorded in tables 1–4. During fasting all dogs received vitamins and salts. When refed, they received a diet of lean meat and sucrose amounting approximately to 66 calories per kg. of body weight. Each normal value is the average of 5 separate observations on 5 dogs. Each thyroidectomized value is the average of 12 separate observations on 6 dogs. Vertical line close to each point represents standard error

t values (8) were calculated in the case of each lipid constituent for *a*, the difference between the mean decrease observed during the 5-day fast of the normal dog and the mean decrease observed for the 5-day fast of the thyroidectomized dog; *b*), the difference between the mean rise observed during the 7-day refeeding of the normal dog and the mean rise observed during the 7-day refeeding of the thyroidectomized dog. These t values are recorded in table 5. t values above the 5 per cent level are not considered significant.

The response of blood lipids in normal and in thyroidectomized dogs to fasting and refeeding have been compared in figure 1 and table 5. A significant difference is demonstrated between the normal and thyroidectomized dog as regards *a*), the decrease in total fatty acids, ester cholesterol and phospholipids produced by fasting and *b*), the rise in total fatty acids and cholesterol esters that occurred with refeeding.

Since it is shown here that the response of the blood lipids to thyroidectomy (i.e. the rise in esterified cholesterol, total fatty acids and to a lesser extent phospholipid) can be completely prevented by fasting or by limitation of the caloric intake, the question arises whether blood cholesterol can be used as a reliable index of thyroid activity, and particularly of hypothyroidism. Apparently it can be of value only when the nutritional state of the animal is known. The results of the present study show that a normal concentration of cholesterol in the blood need not rule out hypothyroidism.

SUMMARY

1. The influence of nutritional state, viz. fasting and feeding, upon the response of cholesterol, total fatty acid, phospholipid and total lipid of the dog to thyroidectomy was investigated.

2. The high levels of blood lipids produced by extirpation of the thyroid glands in the dog are dependent upon nutritional state. The rise of cholesterol esters and total fatty acids can be inhibited by fasting or by limitation of the caloric intake. Refeeding for as short a period as 5 days is sufficient to reestablish high lipid levels in the thyroidectomized dog.

3. Fluctuations in the lipid level of the blood in response to alterations in caloric intake were observed as late as one year after thyroidectomy.

4. The response of blood lipids to alterations in caloric intake is compared in normal and thyroidectomized dogs.

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BLOOD LIPIDS OF THE HYPOPHYSECTOMIZED-THYROIDECTOMIZED DOG[1]

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THE THYROID GLAND exerts a definite control over lipid metabolism. The concentration of blood lipids, especially esterified cholesterol and neutral fat, is elevated after thyroidectomy (1). Although the mechanism whereby these changes are produced is still obscure, it has been shown in this laboratory that the thyroidectomized dog differs from the normal in the degree of response of its blood lipids to alterations in nutritional state (2). Wide variations in the dietary regime of the normal dog produce no marked changes in the concentration of blood lipids (3). Thus, acute fasting for as long as 30 days failed to produce much alteration in the levels of total fatty acids, total cholesterol or of phospholipids of the blood in the normal dog. The experimental production of obesity by which normal dogs were made to increase their weights by as much as 50 to 80 per cent in periods as short as 90 days also failed to produce striking changes in the lipid concentration of the blood (4). In contrast to this relative stability of the blood lipid levels of the *normal* dog, the thyroidectomized dog suffers severe losses of blood lipids during fasting (2). In the absence of the thyroid gland, fasting for as short a period as 5 days can deprive the dog of 50 per cent of the cholesterol esters and 30 per cent of the total fatty acids in its blood. Ths response to refeeding is equally striking in the thyroidectomized dog. The increase in the concentration of blood lipids observed on instituting feeding after a fast can amount to 150 mg. in cholesterol esters and 300 mg. in total fatty acids in as short a period as 7 days (2).

In order to obtain more information on the mechanism whereby thyroidectomy produces its effect on blood lipids, the interrelation of the pituitary gland has been investigated. It was previously found that hypophysectomy *per se* has but little effect on the levels of the blood lipids of the dog (5). The present communication shows that hypophysectomy does not inhibit the rise in the blood lipids produced by thyroidectomy.

EXPERIMENTAL PROCEDURE

Nine adult dogs were used in this investigation. After their admittance to the laboratory they were fed twice daily, at 8:00 A.M. and at 4:00 P.M., a diet of lean meat and sucrose in amounts recorded in tables 1 to 3. Two grams of bone ash were added to each dietary mixture. In addition each dog received with its evening meal

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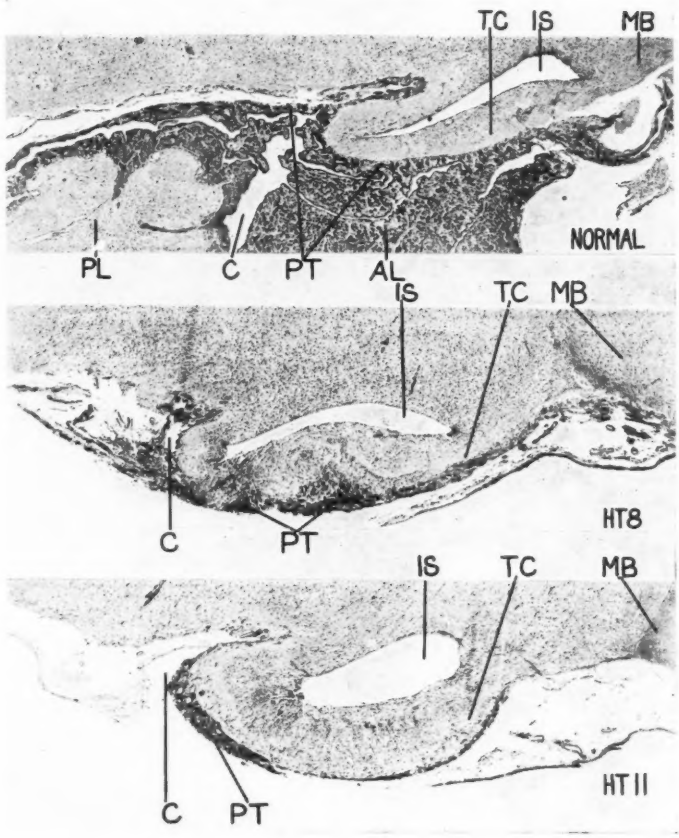

Fig. 1. LONGITUDINAL SECTIONS OF THE BASE OF THE BRAIN OF NORMAL AND OPERATED DOGS (magnification ×30; stain H. and E.). Serial sections were made of the base of the brains of all hypophysectomized-thyroidectomized dogs and these carefully examined for completeness of hypophysectomy. In none of the dogs recorded in this study were anterior lobe cells or posterior lobe cells found. Occasional groups of pars tuberalis cells were found, which in many cases extended into the tuber cinereum. The two hypophysectomized-thyroidectomized sections shown above are typical of the findings. *PL*, posterior lobe; *C*, cleft; *PT*, pars tuberalis; *AL*, anterior lobe; *TC*, tubercinereum; *IS*, infundibular space; *MB*, mammillary body.

5 cc. of a rice bran concentrate (Galen B, Type II liquid[3]), 3 cc. of sardilene oil[4] and 2 gm. of Cowgill's salt mixture (6). The above diet was fed to 8 of the 9 dogs; *dog HT15* received 150 gm. of a whole ground fish (Balto dog food) and 50 gm. of sucrose twice daily. The vitamin concentrates, bone ash and salts were given to all 9 dogs alike.

After a suitable control period, each dog was subjected to complete excision of its pituitary gland. The details of the procedure for hypophysectomy have been de-

[3] This concentrate was generously supplied by The Vitab Corporation, Emeryville, California.

[4] In each gram of this oil was contained a minimum of 100 A.O.A.C. chick units of vitamin D and 600 U.S.P. units of vitamin A.

scribed elsewhere (7, 8). In the procedure employed in this study no cautery was applied to the base of the brain. In most cases the appetites of the dogs were excellent after hypophysectomy.

The dogs were next subjected to complete excision of the thyroid glands. In all cases one or two of the parathyroid glands were preserved with their blood supply intact. All other parathyroid tissue found was transplanted into neighboring muscle. For the first two weeks after the removal of the thyroid glands each dog was fed 5 gm. of calcium lactate and 100 cc. of whole milk per meal in addition to the constituents listed above. In all dogs except *dog HT11*, appetite was lost after removal of the second gland. The exact caloric intake recorded for each dog was maintained, however, by force-feeding. *Dog HT11* ate well for the first 10 months after thyroidectomy; during the last 2 months of its period of observation it was force-fed twice daily.

The completeness of hypophysectomy was established in all 9 dogs at necropsy. Serial sections were made of the base of the brain of each dog and every fifth section carefully examined for hypophyseal tissue. No cells of the anterior, posterior or intermediate lobes of the gland were found in the dogs recorded in this study (see fig. 1). The methods for the sampling and analysis of the blood lipids have been previously described (9). Free cholesterol was determined in an acetone solution from which phospholipids had been precipitated.

RESULTS

The observations presented here were obtained from 9 dogs that were completely deprived of both pituitary and thyroid glands. In all cases the pituitary gland was removed first and at suitable intervals thereafter (36–58 days) both thyroid glands. The concentrations of cholesterol, both free and esterified, phospholipids and of total fatty acids in the blood of these animals were measured during 3 separate intervals. The *first* interval extended from the first day each dog received the diet recorded above until the day on which the hypophysis was excised. This interval, which lasted for 8 to 75 days, provided a *control* period during which the normal lipid level of each dog was determined. The *second* interval extended from the day of hypophysectomy to the day on which the thyroid glands were excised; it lasted from 36 to 58 days. The *third* interval represents the period of survival after excision of both glands. It was the longest period of observation, lasting in one case to 419 days and in 2 other dogs to over one year. The number of blood lipid determinations made during each of the three intervals was not the same in the dogs investigated, but in all of them the largest number of determinations was made during the third interval.

It should be noted here again that, although the caloric intake varied from dog to dog, it was kept rigidly constant during these three periods of observation so far as 8 of the 9 dogs were concerned. The one exception was *dog HT15;* its caloric intake was kept the same during the control period, during the interval between hypophysectomy and thyroidectomy, and during the first 68 days after thyroidectomy. It was modified thereafter. Under the headings of each lipid constituent in the text below all results from 8 of the 9 dogs are dealt with, but from *dog HT15* only those values obtained during the interval from the beginning of the control period until the first 68 days after thyroidectomy are included.

Cholesterol

Control levels. The cholesterol levels of the blood were determined 5 times during the 66 days that *dog HT10* was observed before removal of either glandular tissue.

The 5 values for total cholesterol were strikingly similar; the highest and lowest values were 198 and 190 mg. per 100 cc. of whole blood. In *dog HT8* the cholesterol concentration of the blood was measured on two occasions, on the 7th and on the 73rd day of the control period; despite this interval of 66 days the concentrations of free and esterified cholesterol in the blood on these two occasions were practically the same. Equally good agreement was observed in the three control values of *dog HT3*. As compared with the dogs recorded above, less uniformity was found in the control values of *dogs HT6, HT11* and *HT18*, but even in these animals the highest and lowest values for total cholesterol did not differ by more than 9 per cent. In a single dog (*HT21*) the two control values for esterified cholesterol differed by 27 mg., but the values for free cholesterol were in good agreement.

After hypophysectomy. An interval of 36 to 58 days was permitted to elapse between hypophysectomy and thyroidectomy, during which time 1 to 4 determinations of the lipid concentration of the blood were made. Except for the first 2 weeks, the coloric intake (in respect to both the constituents used and the quantity fed) was not interfered with; each dog received a diet identical with its control diet. The response of blood cholesterol to excision of the hypophysis was not uniform in the 9 dogs examined. In 6 of them the levels of esterified cholesterol were found definitely above those of the preceding control period. In none of the hypophysectomized dogs, however, did the levels of total cholesterol rise above the highest recorded for normal dogs subjected to overfeeding (4).

The cholesterol concentration in the blood of *dog HT6* was determined twice after hypophysectomy, at 27 and 42 days; on neither occasion did the levels of total, free and ester cholesterol differ markedly from previously established control levels. The blood of *dog HT11* was examined only once after excision of the pituitary gland (30 days), at which time the concentration of cholesterol (both free and esterified) was not affected by hypophysectomy. The levels of cholesterol found in *dog HT21* after hypophysectomy were not significantly above its control values.

Increased levels of esterified cholesterol were found after hypophysectomy in *dogs HT3, HT8, HT10, HT18, HT22* and *HT15*. As early as 10 days after complete excision of the pituitary gland, a rise in cholesterol esters was observed in *dog HT15*. The values for cholesterol esters found at 20, 29 and 42 days after hypophysectomy were respectively 100, 118 and 120 mg. per 100 cc. of whole blood. The control values for this lipid constituent were 34 to 43 mg. On the single occasion when the blood of *dog HT3* was examined after hypophysectomy, cholesterol esters had risen from control levels of 63–69 to 150 mg. On the 44th day after hypophysectomy the concentration of cholesterol esters in the blood of *HT8* was approximately twice that of the control period. Although the levels of cholesterol esters in *dogs HT18* and *HT22* did not rise above 82 mg., these values nevertheless represent an increase above their respective control values.

In none of the 9 dogs studied did a significant increase in free cholesterol occur after hypophysectomy. This is well brought out by dog *HT15*, in which the 3 control values ranged from 127 to 130, whereas the 4 values obtained at intervals of 10, 20, 29 and 42 days after complete excision of the pituitary fluctuated from 120 to 129 mg.

After excision of both hypophysis and thyroid. It was noted above that hypophysectomy failed to increase the concentration of free cholesterol in the blood of any of the 9 dogs studied. Control values were obtained on 26 occasions in these 9 animals, and only a single value for this lipid constituent was above 130 mg. per 100 cc. of whole blood. The free cholesterol content of the blood was measured 21 times in the

TABLE 1. EFFECT OF HYPOPHYSECTOMY AND THYROIDECTOMY ON WHOLE BLOOD LIPIDS OF DOGS HT3, HT6, HT8 AND HT10

(All lipid values expressed as mg. per 100 cc. of whole blood)

Dog	Weight	Period of Observation					Cholesterol			Total Fatty Acids	Phospholipid	Total Lipids
		Total days	After hypophysectomy	After thyroidectomy	Diet per day		Total	Free	Ester			
					Meat	Sucrose						
	kg.		days	days	gm.	gm.						
HT3 ♀	8.5	14	Before	Before	220	44	160	97	63	362	352	512
	9.0	56	Before	Before	220	44	170	101	69	372	418	542
	9.1	70	Before	Before	220	44	167	103	64	389	432	556
	10.1	99	26	Before	220	44	246	96	150	545	304	791
	11.0	134	61	3	220	44	208	107	101	409	385	617
	11.2	149	76	18	220	44	267	138	129	639	427	906
	10.6	170	97	39	220	44	284	129	155	629	460	913
	10.7	201	128	70	220	44	265	144	121	580	397	845
HT6 ♂	9.4	0	Before	Before	270	54	152	95	57	378	307	530
	9.5	7	Before	Before	270	54	152	106	46	330	324	482
	9.5	14	Before	Before	270	54	152	93	59	350	371	502
	10.2	56	Before	Before	270	54	144	96	48	378	361	522
	10.2	70	Before	Before	270	54	158	108	50	384	368	542
	11.2	99	27	Before	270	54	150	106	44	349	333	499
	11.2	115	42	Before	270	54	156	97	59	352	322	508
	11.5	134	62	16	270	54	214	100	114	533	423	747
	11.7	139	67	21	270	54	262	132	130	570	362	832
	12.3	149	77	31	270	54	294	97	197	508	—	802
	13.1	168	96	50	270	54	224	117	107	525	445	749
	14.1	201	129	83	270	54	394	149	245	597	502	991
	15.3	244	162	126	270	54	430	181	249	682	615	1112
HT8 ♀	8.0	7	Before	Before	250	50	154	108	46	371	398	525
	8.8	73	Before	Before	250	50	144	103	41	378	391	522
	9.8	103	28	Before	250	50	180	109	71	379	330	559
	10.3	119	44	Before	250	50	193	84	109	464	274	657
	11.2	138	63	16	250	50	421	173	248	722	469	1143
	11.7	153	78	31	250	50	369	138	231	620	—	989
	12.5	172	97	50	250	50	435	168	267	790	496	1225
	13.7	205	130	83	250	50	334	161	173	705	605	1039
	14.5	248	173	146	250	50	475	190	285	811	684	1286
	15.2	288	213	186	250	50	532	228	304	698	582	1230
	15.2	460	385	358	250	50	311	134	177	610	501	921
	15.5	495	420	393	250	50	347	148	199	710	486	1057
	16.5	505	430	403	250	50	244	118	126	600	377	844
	16.5	521	446	419	250	50	267	134	133	620	418	887
HT10 ♀	8.6	0	Before	Before	260	52	190	110	80	399	364	589
	9.0	7	Before	Before	260	52	194	103	91	476	496	670
	9.3	14	Before	Before	260	52	198	113	85	402	428	600
	9.5	56	Before	Before	260	52	190	123	67	430	433	620
	9.7	66	Before	Before	260	52	190	125	65	424	453	614
	10.7	99	30	Before	260	52	202	130	72	371	400	573
	11.3	115	46	Before	260	52	236	128	108	480	462	716
	12.0	134	65	16	260	52	331	160	171	743	568	1074
	12.7	149	80	31	260	52	420	175	245	627	612	1047
	13.1	168	99	50	260	52	327	164	163	743	615	1070
	14.0	201	132	83	260	52	390	157	233	753	524	1143
	15.1	244	175	126	260	52	278	138	140	744	566	1022
	15.2	274	205	156	260	52	395	122	273	802	512	1197
	15.5	284	215	166	260	52	410	171	239	561	593	971
	16.7	326	257	208	260	52	307	158	149	697	620	1004
	18.0	395	326	277	260	52	313	160	153	655	597	968
	18.7	431	362	313	260	52	301	158	143	731	572	1032
	20.0	494	425	376	260	52	211	126	85	541	482	752
	20.6	516	447	398	260	52	241	112	129	580	443	821

same dogs after hypophysectomy; the highest value observed after the excision of the pituitary gland was 130 mg. After the removal of the thyroid gland, an increase in the level of this lipid constituent occurred in *all* dogs. Values of 180 mg. and over were observed in *dogs HT6, HT8, HT11, HT15* and *HT21*. In two dogs the free cholesterol content of the blood after excision of both hypophysis and thyroid glands rose to over 200 mg.

Definite increases in free cholesterol were observed as early as 15 days after excision of the second gland in *dog HT15*. In the other animals, however, values of 180 mg. and over were found after much longer intervals had elapsed.

Three types of response were found in esterified cholesterol: *a*), No change in the concentration of this lipid constituent in the blood of *dogs HT6* and *HT11* occurred after hypophysectomy. However, after excision of the second gland (i.e. thyroid) definite increases in esterified cholesterol occurred in both dogs. The levels of ester cholesterol more than doubled in both dogs as early as 16 days after removal of the thyroid gland, and values over 240 mg. were found in *HT6* on the 83rd day and on the 126th day in *dog HT11*.

b), In *dog HT3* the total period of observation after thyroidectomy was 70 days. In this dog, an increase in cholesterol esters was observed after hypophysectomy; this increased level was maintained after thyroidectomy. None of the 4 values obtained after thyroidectomy was below 100 mg.

c), The third type of response deals with those dogs in which an increase in cholesterol esters was observed after hypophysectomy and a further increase (and in many cases a very marked increase) after thyroidectomy. This type of response was found in 6 of the 9 dogs reported here.

The short interval in which the cholesterol ester concentration of the blood rose to values above 200 mg. is worthy of note. Sixteen days after thyroidectomy a value of 248 was obtained for this lipid constituent in *dog HT8*; in *dog HT10* it rose to 245 mg. in 31 days.

The highest value for cholesterol esters observed in this series of 9 dogs was 304 mg. The highest control value was 91 mg. In *dog HT21* a value of 298 was observed 195 days after the second gland was excised.

It was stated above that the highest value for total cholesterol in obese normal dogs was 241 mg. In 7 of the 9 dogs subjected to excision of both pituitary and thyroid glands, values for total cholesterol over 400 mg. per 100 cc. of whole blood were not uncommon findings. The highest value was observed in *dog HT15*, in which total cholesterol rose to 560 mg.

In table 4, the increases represented by the maximum blood cholesterol values in the 9 hypophysectomized-thyroidectomized dogs have been expressed as percentage change relative to *a*), their respective highest control values and *b*), the highest values observed in normal dogs. Increases of as much as 560 per cent were observed in the case of cholesterol esters and 156 per cent for free cholesterol.

Total Fatty Acids

Control levels. It is shown in tables 1 to 3 that under the standardized dietary conditions of the present investigation a constant level of total fatty acids can be maintained in the blood of the normal dog. Thus the three values in *dog HT3* obtained 14 56 and 70 days after it was placed on the experimental diet were respectively 362, 372 and 389 mg. The control period in *dog HT6* lasted for 70 days, during which blood was examined 5 times for its total fatty acid content; the 5 values ranged from 330 to 384 mg. The closest agreement was observed in *dog HT15;* in this animal the three values obtained during the 3 control days (i.e. between the day it was placed on

TABLE 2. EFFECT OF HYPOPHYSECTOMY AND THYROIDECTOMY ON WHOLE BLOOD LIPIDS OF DOGS HT11, HT18, HT21 AND HT22

(*All lipid values expressed as mg. per 100 cc. of whole blood*)

Dog	Weight	Period of Observation					Cholesterol			Total Fatty Acids	Phospholipid	Total Lipid
		Total days	After hypophysectomy	After thyroidectomy	Diet per day		Total	Free	Ester			
					Meat	Sucrose						
	kg.		days	days	gm.	gm.						
HT11 ♀	6.0	0	Before	Before	180	36	178	114	64	400	386	578
	6.6	7	Before	Before	180	36	161	105	56	396	355	557
	6.8	38	30	Before	180	36	175	109	66	380	302	555
	7.8	73	65	16	180	36	248	113	135	493	342	741
	8.3	88	80	31	180	36	310	145	165	567	440	877
	9.0	109	101	52	180	36	306	126	180	662	460	968
	9.3	140	132	83	180	36	323	145	178	607	559	930
	10.5	183	175	126	180	36	405	153	252	649	563	1054
	10.7	223	215	166	180	36	304	145	159	702	477	1006
	12.0	347	339	290	180	36	276	131	135	848	626	1124
	12.2	395	387	338	180	36	331	164	167	688	589	1019
	12.5	431	423	374	180	36	406	180	226	702	530	1108
HT18 ♂	10.2	9	Before	Before	325	10	173	142	31	392	351	565
	10.7	16	Before	Before	325	10	162	123	39	351	396	513
	11.9	60	24	Before	325	10	178	129	49	396	362	574
	11.9	74	38	Before	325	10	208	127	81	405	478	613
	12.1	82	46	Before	325	10	190	122	68	395	393	585
	13.0	99	53	17	325	10	206	106	100	505	366	711
	14.7	127	81	45	325	10	246	143	103	724	487	970
	15.3	152	106	70	325	10	200	124	76	656	377	856
	16.9	177	131	95	325	10	313	147	166	656	489	969
	18.0	214	168	132	325	10	412	175	237	638	493	1050
HT21 ♀	7.9	9	Before	Before	260	10	137	99	38	356	314	493
	7.8	16	Before	Before	260	10	164	102	62	356	361	520
	7.5	60	23	Before	260	10	187	112	75	435	415	622
	8.0	74	37	Before	260	10	202	130	72	462	472	668
	7.8	82	45	Before	260	10	132	104	28	308	—	440
	8.1	99	62	17	260	10	306	145	161	515	473	821
	9.0	127	90	45	260	10	261	151	110	689	448	950
	10.0	152	115	70	260	10	333	141	192	690	476	1023
	11.0	177	140	95	260	10	324	154	170	871	551	1195
	11.7	214	177	132	260	10	326	191	135	758	562	1084
	13.5	277	240	195	260	10	446	148	298	711	585	1157
	14.5	299	262	217	260	10	393	159	234	740	522	1133
HT22 ♀	8.5	7	Before	Before	270	10	162	120	42	392	376	554
	8.4	12	0	Before	270	10	141	118	23	361	398	502
	8.1	35	23	Before	270	10	153	102	51	390	390	543
	8.5	49	37	Before	270	10	198	116	82	416	443	614
	8.8	57	45	0	270	10	183	119	64	362	405	545
	9.5	74	62	17	270	10	193	80	113	459	442	652
	10.4	102	90	45	270	10	240	125	115	526	407	766
	11.2	125	113	68	270	10	264	130	134	468	396	732
	12.0	150	138	93	270	10	283	146	137	558	492	841
	13.0	187	175	130	270	10	304	149	155	437	415	741
	14.5	250	238	193	270	10	243	139	104	534	434	777
	18.3	341	329	284	270	10	203	123	80	504	451	707
	18.8	353	341	296	270	10	230	130	100	485	435	715
	19.7	368	356	311	270	10	265	147	118	557	509	822

the diet until the removal of the pituitary gland) were 360, 368 and 368 mg. per 100 cc. of whole blood. The highest control value was found in dog *HT*10, namely 476 mg.

After hypophysectomy. Definite increases in the total fatty acid content of the blood were observed in only 2 of the 9 dogs, namely *HT3* and *HT15*. In the former the total fatty acid content of the blood on the 26th day after hypophysectomy was 545 mg., as compared with its highest control value of 389 mg. In dog *HT15*, 4 determinations were made, 10, 20, 29 and 42 days after excision of the pituitary gland; the earliest as well as the last values were well above the control levels.

TABLE 3. EFFECT OF HYPOPHYSECTOMY AND THYROIDECTOMY ON WHOLE BLOOD LIPIDS OF DOG HT15
(*All lipid values expressed as mg. per 100 cc. of whole blood*)

Weight	Period of Observation		Diet per Day		Days on Diet	Cholesterol			Total Fatty Acids	Phospholipid	Total Lipid
	After hypophysectomy	After thyroidectomy	Fish	Sucrose		Total	Free	Ester			
kg.	*days*	*days*	*m.*	*m.*							
6.5	Before	Before	300	100	14	168	128	40	360	330	528
6.8	Before	Before	300	100	22	164	130	34	368	350	532
6.6	Before	Before	300	100	32	170	127	43	368	341	538
7.4	10	Before	300	100	45	195	129	66	498		693
8.1	20	Before	300	100	55	227	127	100	430		657
9.1	29	Before	300	100	64	242	124	118	528		770
8.9	42	Before	300	100	77	240	120	120	523	315	763
9.5	62	15	300	100	97	290	153	137	796	549	1086
9.6	69	22	300	100	104	332	158	174	532	475	864
10.3	81	34	300	100	116	336	167	169	545		881
11.1	91	44	300	100	126	300	163	137	848	480	1148
12.1	113	66	300	100	148	416	241	175	1040	638	1456
12.3	114	67	300	100	149	496	235	261	1950	752	2446
12.3	115	68	300	100	150	488	262	226	2220	705	2708
12.0	128	81	150	50	7	560	333	227	1518	522	2078
12.1	149	102	150	50	28	424	234	190	1406	524	1830
12.1	155	108	150	50	34	275	216	59	977	350	1252
12.0	163	116	150	50	42	400	225	175	1326	575	1726
12.1	164	117	150	50	43	372	204	168	1356	400	1728
10.4	205	158	54	10	41	220	145	75	438	333	658
9.7	235	188	54	10	71	182	162	20	405	299	587
9.7	245	198	36	6	10	168	131	37	410		578
9.0	271	224	36	6	36	148	117	31	373	313	521
11.5	326	279	150	50	41	228	132	96	670	428	898
11.5	332	285	150	50	47	258	145	113	582	540	840
11.0	340	293			8	258	151	97	646	347	894
10.5	351	304			19	248	178	89	610	327	877
9.3	353	306			21	267	185	61	599	352	845
9.0	357	310			25	246	149	39	443	296	631

In 5 dogs (*HT6*, *HT10*, *HT11*, *HT18* and *HT22*) the values for total fatty acids found after hypophysectomy were well within the range of the control values.

In 2 dogs (*HT8* and *HT21*) the responses were irregular. The first two results in *HT21*, obtained at 23 and 37 days after removal of the hypophysis, showed an increase, but a later determination obtained on the 45th day after hypophysectomy was below the control range. The first lipid determination after hypophysectomy in dog *HT8* was made on the 28th day and was not above the control level; the one obtained on the 44th day, however, was approximately 90 mg. above the control level.

After hypophysectomy and thyroidectomy. There can be no doubt of the nature of the response of total fatty acids after the second glandular tissue was removed. There was not a single animal in which total fatty acids did not rise after thyroidectomy, and in those cases in which small increases occurred after hypophysectomy, the additional removal of the thyroid gland led to a much more pronounced increase in this lipid

constituent. The 9 dogs in this study were kept alive for variable periods after excision of both glandular tissues, during which time numerous determinations of blood lipids were made. All values for total fatty acids obtained in each dog deprived of both thyroid and pituitary glands were well above their respective control values. This was true in the case of the determinations made in the early intervals as well as in those made a year or more after the removal of the second gland.

The most spectacular rise in total fatty acids occurred in *HT15*. In this dog total fatty acids rose to the extraordinary value of 2220 mg., a 4-fold increase over the control value. In the 8 other dogs used in this study the maximum value found in each do was below 1000 mg. Values of 800 or over were observed in *HT8*, *HT10*, *HT11* and *HT21*.

TABLE 4. PERCENTAGE CHANGE REPRESENTED BY MAXIMUM LIPID VALUES OBSERVED IN HYPOPHYSECTOMIZED-THYROIDECTOMIZED DOGS

Dog Number	Above Highest Control Value				Above Highest Value Observed in Obese Normal Dogs			
	Cholesterol		Total fatty acids	Phospholipids	Cholesterol		Total fatty acids	Phospholipids
	Free	Ester			Free	Ester		
HT3	25	125	64	7	4	99	25	−8
HT6	68	278	78	66	46	218	34	24
HT8	111	560	115	72	84	290	59	38
HT10	40	169	69	25	41	214	57	25
HT11	58	254	112	62	45	190	67	24
HT15	156	89	322	115	169	191	336	52
HT18	30	430	84	25	41	204	42	−1
HT21	87	380	117	62	54	282	71	18
HT22	24	222	42	28	20	99	9	−2

The speed of the response is shown by the fact that in 8 of the 9 dogs here studied marked increases occurred at the earliest interval after thyroidectomy at which lipid determinations were made, namely 15 to 17 days. In *HT15* the total fatty acid content of the blood rose from 523 to 796 mg. in the first 15 days that elapsed after excision of the thyroid gland. At the end of the first 16 days after thyroidectomy total fatty acids rose from 480 to 743 in dog *HT10*.

The highest total fatty acid values observed in the 9 hypophysectomized-thyroidectomized dogs studied here represent increases of 42 to 322 per cent above their respective control values, or 9 to 336 per cent above the highest value observed in normal obese dogs (table 4). As a rule the increases in total fatty acids were not as great as in cholesterol esters.

Phospholipids

After hypophysectomy. In 7 of the 9 dogs, the phospholipid content of the blood was not elevated by hypophysectomy. None of the values for this lipid constituent observed after excision of the pituitary gland in 6 of the dogs (*HT3*, *HT6*, *HT8*, *HT10*, *HT11* and *HT15*) was above its respective control value. The results obtained in 2 dogs (*HT18* and *HT22*) were not consistent. The phospholipid content of the blood in these 2 dogs was above their respective controls on the 37th day after hypophysectomy, but on two other occasions, namely 24 and 46 days after hypophysectomy, the values were well within the range of their controls. In a single dog (*HT21*) both phospholipid values obtained after hypophysectomy were above those of the preceding control period.

After hypophysectomy and thyroidectomy. The results taken as a whole show that significant increases in the phospholipid content of the blood occur in dogs deprived of both pituitary and thyroid glands. In *HT8* the highest value for phospholipid was 684 mg., the control values 391 to 398 mg. The highest control value in *dog HT10* was 496 mg.; of the 12 lipid determinations made on this dog after removal of the thyroid gland, 5 of them were close to, or over, 600 mg. Similar changes were observed in *HT6*, *HT11* and *HT21;* phospholipid values of over 550 were frequently observed in these dogs after they were deprived of both glandular tissues. The most spectacular response was obtained in *dog HT15:* the phospholipid content of the blood rose to 752 mg. on the 67th day after removal of the second gland. In only one dog (*HT3*) did a questionable response of phospholipid occur after the second operation.

The changes produced by excision of both hypophysis and thyroid tissues appear to differ in respect to total fatty acid and phospholipid. Although (as noted above) the concentrations of these two lipid constituents are elevated in the blood of dogs deprived of both glands, the rise in phospholipid is not so regular as that in total fatty acids. There also appears to be a difference in the time of onset of the rises; that of phospholipid is somewhat later than that of total fatty acid. Table 4 shows that the extent of the increases in blood phospholipid that take place in the hypophysectomized-thyroidectomized dogs are less pronounced than those in cholesterol esters and total fatty acids.

Total Lipid

The values for total lipid are obtained by the addition of total cholesterol and total fatty acids. In order to assess the significance of the levels in total lipid attained after excision of both pituitary and thyroid glands, the following three lipid values should be noted: *a*), the highest control value for total lipid observed in the 9 dogs of the present study was 670 mg.; *b*), the highest value found in this group of dogs after complete excision of only the pituitary gland was 791 mg.; *c*), as was reported elsewhere, the maximum total lipid value in normal dogs subjected to over-feeding was 773 mg. per 100 cc. of whole blood.

After excision of both pituitary and thyroid glands, maximum values well over 1000 mg. were found in 7 of the 9 dogs recorded here. In the two dogs (*HT3* and *HT22*) in which total lipid of the blood did not reach a concentration of 1000 mg., maximum values of 913 and 841 mg. were nevertheless observed. The highest value for total lipid was found in *dog HT15;* it was 2708 mg.; this amount of total lipid was present on the 68th day after complete removal of the thyroid gland, by which time the dog had already been deprived of its hypophysis for 115 days.

Weight Changes in Dogs Subjected to Removal of both Hypophysis and Thyroid Gland

It has been observed repeatedly in this laboratory that the daily ingestion of 30 gm. of lean meat plus 6 gm. of sucrose[5] per kg. provides sufficient calories for maintenance of weight in normal adult dogs kept at 25° C. in cages measuring 3×4 ft. This represents a caloric intake of approximately 60 calories per kg. Thus 18 dogs whose initial weights varied from 6 to 14.0 kg. showed little or no increase in weight when fed the above amounts of lean meat and sucrose daily for 20 to 30 days (10). Two dogs weighing 7.3 and 7.7 kg. were fed this diet for 100 days; at the end of this period their weights were 7.7 and 10.5 kg. respectively. In only 2 of a total of 40 dogs studied were losses of about 10 per cent in weight observed when they were fed daily a dietary mixture of 30 gm. of lean meat plus 6 gm. of sucrose per kg.

[5] Vitamins and salts always included in diets.

Dogs HT3, HT6, HT8, HT10 and *HT11* received a diet of 30 gm. of lean meat plus 6 gm. of sucrose per kg. of initial body weight.[6] The control period (i.e., the interval between the time the dog was first placed on the diet until hypophysectomy) lasted for 69 days or more in the first 4 of these dogs; at the end of this period their weights were above those found at the start of the experiment. The control period for *HT11* was of shorter duration. Its weight was maintained by the amounts of meat and sucrose fed.

Dogs HT18, HT21 and *HT22* received 33 gm. of lean meat per day per kg. of initial body weight plus 10 gm. of sucrose per day, irrespective of body weight. This diet served to hold their weights at initial levels throughout the control period.

After the excision of both glands most of the dogs showed a loss of appetite. Each dog, however, received the full diet recorded in the tables. All food not voluntarily ingested was force-fed. As a rule each dog was fed twice daily. In order to ensure complete ingestion of the diets without subsequent vomiting, it was sometimes found necessary to force-feed the amounts specified in the tables in 4 equal parts during the day. When necessary, this was done at intervals between 7:00 A.M. and 5:00 P.M.

The gain in weight that occurred in all dogs after excision of both hypophysis and thyroid was to be expected under the conditions of the present investigation. A number of the dogs more than doubled their initial weights.[7] At the start of its control period *dog HT8* weighed 8.0 kg.; 419 days after removal of both glandular tissues its weight was 16.5 kg. *Dogs HT10* and *HT11* more than doubled their weights after being deprived of hypophysis and thyroid gland for a little over a year. A pronounced gain in weight also occurred in *dog HT15*. Its caloric intake was kept high enough to double its weight as early as 150 days after being placed on the diet; at this interval it had been thyroidectomized for 68 days.

This gain in weight should not be confused with growth. All dogs employed were fully grown adult dogs. The energy expenditure (B.M.R.) of each dog is reduced in the absence of thyroid and hypophysis glands. Yet the caloric intake was kept at normal levels, i.e., at levels that maintained or even increased their weights when normal. It was for this reason that the considerable gain in weight in the hypophysectomized-thyroidectomized dogs was expected. At postmortem examination huge deposits of fat were found in them.

Effect of Alteration in Caloric Intake upon Blood Lipids of the Hypophysectomized-Thyroidectomized Dog

The influence of reduction in the caloric intake was determined in a single animal, *HT15*. For the first 150 days of observation it was maintained on a constant daily intake of 300 gm. of whole ground fish and 100 gm. of sucrose. This interval included 35 control days, 47 days after hypophysectomy and 68 days after thyroidectomy. The blood lipids rose in this dog, as already noted above, and on the 149th and 150th days of observation the total lipid content of the blood was 2446 and 2708 mg.

For the next 49 days the animal received daily 150 gm. of the fish and 50 gm. of sucrose. Although this diet amounted to half of its preceding intake, it had practically no effect on the dog's weight. During this period 5 blood lipid determinations were made; none of the values for total lipid were below 1000 mg.

For the next 71 days the daily intake was reduced to 54 gm. of fish and 10 gm. of

[6] Initial weights not recorded for all dogs.

[7] It should be noted here that by overfeeding, normal dogs can be made to increase their weights by as much as 80 per cent in 3 months (4).

sucrose. This diet brought about a reduction in the dog's weight and with it a pronounced fall in the concentration of all lipid constituents in the blood. Two lipid determinations were made during these 71 days, the first on the 41st day and the second on the last day. On the 41st day the dog's weight was found reduced from 12.1 to 10.4 kg., and the content of total lipids had dropped from 1728 to 638 mg. This drop in total lipids was reflected in the other constituents, particularly total fatty acids, which in 41 days of the reduced diet fell from 1356 to 438 mg. The dog's weight and the lipid content of its blood continued to fall while it was kept on 54 gm. of fish and 10 gm. of sucrose. On the 71st day the content of cholesterol, phospholipid and total lipid had returned to original control levels.

For the next 36 days the intake was further reduced; *dog HT15* now received 36 gm. of fish and 6 gm. of sucrose daily. This diet effected a further reduction weight, without, however, producing further striking changes in the lipid concentration of the blood. At the end of the 36 days the values for total cholesterol, total fatty acid, phospholipid and total lipid were respectively 148, 373, 313 and 521, as compared with control values of 170, 368, 341 and 538 mg. This failure to alter further the level of the blood lipids was to be expected, since it had already been reduced to approximately normal levels by the diet of 54 gm. of fish and 10 gm. of sucrose.

For the next 47 days the caloric intake of this dog was increased to 150 gm. of fish and 50 gm. of sucrose. A pronounced increase in all lipid constituents was observed at the end of this period of observation.

DISCUSSION

The importance of maintaining an adequate caloric intake in order to obtain high lipid levels after thyroidectomy was pointed out in the preceding paper (10). Fasting or limiting the caloric intake led to a severe depletion in the lipid concentration in the blood of the thyroidectomized dog. For this reason the hypophysectomized-thyroidectomized dogs in the present study were fed a diet sufficient for their preoperative needs, even though their caloric expenditure (B.M.R.) had been greatly reduced by excision of both glands. The increase in weight that was deliberately produced by such dietary treatment raised a question of the rôles of 3 factors: *a*), altered nutritional state, *b*), thyroid gland and *c*), pituitary gland, in the production of the extraordinary increases observed in the lipid content of the blood during the interval after the dogs were deprived of both pituitary and thyroid glands.

Although, as noted in the case of *dog HT15*, a suitable caloric intake is just as necessary to obtain a high lipid concentration in the blood of dogs subjected to both hypophysectomy and thyroidectomy as it is in dogs subjected to thyroidectomy only, it can nevertheless be demonstrated that the immense blood lipid levels observed in dogs deprived of both pituitary and thyroid glands are not the result of their change in weight. Thus in normal dogs that had gained as much as 80 per cent of their inital weight by force-feeding a high caloric diet for 90 days, the highest value found for total lipid was of the order of 773 mg. and for total cholesterol 241 mg. By the time that the hypophysectomized-thyroidectomized dogs had shown comparable gains in weight, their values for total lipid were well over 1000 mg. and those for total cholesterol over 400 mg. This comparison between normal and hypophysectomized-thyroidectomized dogs leaves no doubt that nutritional state—although a necessary accompaniment—is not the sole factor in the production of hyperlipemia in dogs deprived of both pituitary and thyroid glands.

The relative rôle of the thyroid and pituitary glands in the production of hyperlipemias remains to be considered. All dogs were first hypophysectomized, and rather

long intervals were allowed to elapse between hypophysectomy and thyroidectomy. The earlier finding that abnormal increases in all blood lipid constituents do not occur after hypophysectomy (5) is confirmed by the observations made here. The removal of the hypophysis produced no significant change in the blood content of total fatty acid, phospholipid and free cholesterol, although an irregular increase did occur in esterified cholesterol. The abrupt and striking rise in blood lipid that occurred after removal of the second gland, as compared with the irregular response observed after hypophysectomy alone, suggests that thyroid deficiency is the primary stimulus. Indeed, it is by no means unlikely that such changes as did occur in the esterified cholesterol content of the blood during the interval between hypophysectomy and thyroidectomy were not due directly to loss of hypophyseal tissue but resulted from thyroid atrophy induced by excision of the pituitary gland.

The results of the present investigation show that the rise in all lipid constituents of the blood that follows thyroidectomy can occur in the absence of all hypophyseal tissue. Moreover, the increases in the total fatty acid, phospholipid and free and esterified cholesterol content of the blood observed in the 9 hypophysectomized-thyroidectomized dogs of the present study are just as pronounced as those previously reported in thyroidectomized dogs, if not more so. If, as it now appears, the content of blood lipids is controlled, in part at least, by the level of the thyroid hormone, a more pronounced rise in blood lipids might be expected when both glands are excised than when the thyroid gland alone is removed. Accessory thyroid tissue, if present, would be depressed by the additional removal of the hypophysis, and in this way the dog deprived of both glands would yield a more exaggerated picture of the effects of deficiency in thyroid hormone than the dog reprived of thyroid gland only. Although other possibilities have not been ruled out, this may explain the more uniformly high blood lipid levels of the hypophysectomized-thyroidectomized dog as compared with those of the previously reported thyroidectomized dogs (16).

The results of the present investigation are at variance with those reported by Thompson and Long, who state that the hypercholesterolemia of the thyroidectomized dog is abolished by hypophysectomy (11). The dogs employed by these workers were thyroidectomized before hypophysectomy, whereas in the dogs recorded here the glands were excised in the reverse order. It is hardly likely, however, that the order in which glands were removed can explain the differences in the two studies, for the end result is the same, an animal deprived of both thyroid and hypophyseal tissues. The possibility that food intake may explain the failure of the blood cholesterol of Thompson and Long's dogs to remain elevated after hypophysectomy should be considered.

SUMMARY

1. The content of free and esterified cholesterol, phospholipids and total fatty acids in whole blood of dogs subjected to excision of both hypophysis and thyroid glands was investigated. The hypophysis was excised first, after which 3 weeks were allowed to elapse before removal of the thyroid gland. The completeness of hypophysectomy was established at necropsy in all 9 dogs recorded here. The total period of observation extended from 201 to 516 days; for 70 to 419 days of this period the dogs were deprived of both hypophyseal and thyroid tissue. In 3 of the dogs the lipid levels of the blood were examined at repeated intervals for well over one year after both glands had been removed. Although the diets received by all 9 dogs were not the same, in the case of each dog the same diet was fed daily during 3 intervals: *a*), the control period, *b*), the interval between hypophysectomy and thyroidectomy and *c*), after thyroidectomy. In this way each dog received a constant caloric intake

during these 3 periods of observation; the caloric intake was sufficient for weight maintenance before excision of the glands, but more than sufficient after the dogs were deprived of pituitary and thyroid glands.

2. The concentration of all lipid constituents is markedly increased in the blood of the hypophysectomized-thyroidectomized dogs. In none of the 9 dogs examined did the levels of free and ester cholesterol, phospholipid and total fatty acid fail to increase after both glands had been excised.

3. The uniformly striking and rapid increases in the concentration of blood lipids that occurred after removal of the second gland (i.e. the thyroid gland), as compared with the slight and irregular response after hypophysectomy alone, suggest that an acute deficiency in the thyroid hormone *per se* is responsible for the high concentration of the blood lipids in the hypophysectomized-thyroidectomized dog.

4. The rôle of caloric intake in the production of the hyperlipemia of the hypophysectomized-thyroidectomized dog is pointed out.

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INFLUENCE OF CERTAIN STEROID HORMONES ON DIFFUSION OF CHLORIDE INTO PERITONEAL SPACE[1]

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IN AN EARLIER STUDY (1) we demonstrated that the administration of desoxycorticosterone acetate or progesterone to dogs results in a marked increase in the rate of entrance of sodium and chloride into 5.5 per cent dextrose solution introduced into the peritoneal cavity. These observations appeared to support the hypothesis that the effect of the hormones upon the distribution of these electrolytes is exerted through an influence upon membranes in general and that their action in decreasing renal elimination of sodium and chloride may constitute a part of the more generalized action or may perhaps be secondary to extra-renal phenomena. In the present study, we have similarly tested the effect of other steroid hormones since there is some evidence that the influence on sodium and potassium excretion in the urine is shared by a number of steroid and related hormones. A number of preliminary experiments in rabbits suggested the existence of an important species difference in this regard; therefore, further experiments of a similar nature were made in these animals. The effect of pregnancy on this phenomenon was also studied in three rabbits.

METHODS

A solution of 5.5 per cent dextrose in distilled water, 100 cc. per kg. of body weight, was instilled in the peritoneal cavity of normal unanesthetized dogs and rabbits, 10 minutes being allowed for the instillation. Samples of fluid were removed by syringe after manipulation of the abdomen and repeated withdrawal and reinjection to ensure uniform composition of the fluid. These were taken 5, 10, and 15 minutes after completion of the intraperitoneal instillation since, in our earlier experiments, it was noted that the most significant changes occurred within this period. Chloride determinations were made by the method of Wilson and Ball (2). The animals received intramuscular injections of the hormones in oily solution at specified intervals before the introduction of fluid into the peritoneum.

RESULTS

A summary of the findings is presented in table 1. The results with desoxycorticosterone acetate and progesterone in the dogs have been reported previously (1) but are included in the table for purposes of comparison.

It will be noted that, in the dog experiments, with the three additional hormones studied, namely, diethylstilbestrol, α-estradiol benzoate, and testosterone propionate, the average concentrations of chloride in the peritoneal fluid are much higher than in the control animals and are of approximately the same magnitude as the average values

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for desoxycorticosterone acetate and progesterone. In every instance, the minimum values in the treated series were greater than the highest value in the control group. Alpha-estradiol benzoate was the most effective agent in hastening the passage of chloride into the artificial peritoneal fluid.

In the rabbit experiments, no significant change in the rate of diffusion of chloride

TABLE 1. INFLUENCE OF STEROID HORMONES ON PASSAGE OF CHLORIDE INTO THE PERITONEAL FLUID

	Controls	Desoxycorticosterone acetate	Progesterone	Diethylstilbestrol	Estradiol benzoate	Testosterone propionate,	Pregnant
Dog Experiments							
No. of Animals	11	9	3	3	3	3	
Dose Range, mg.		4–20	15	5	5	25	
Time Range, hr.		17½–72	21½–72	24	24	24	
Peritoneal Fluid= Cl., mg. per 100 cc.							
5 Minutes							
Range	17–30	39–67	48–75	44–70	70–90	40–44	
Average	25	51	60	61	83	43	
10 Minutes							
Range	32–52	61–100	85–102	80–102	96–130	62–96	
Average	44	78	92	91	118	62	
15 Minutes							
Range	39–67	83–130	107–125	115–140	110–168	82–160	
Average	55	97	116	131	145	131	
Rabbit Experiments							
Number of Animals	6	4	3	3	4		3
Dose Range, mg.		5	1–10	1–10	2.5		
Time Range, hr.		24–48	24–48	24–48	24		
Peritoneal Fluid= Cl., mg. per 100 cc.							
5 Minutes							
Range	24–50	28–32	28–30	30–36	44–66		36–49
Average	40	30	29	34	59		42
10 Minutes							
Range	36–74	38–42	38–42	38–46	54–90		48–66
Average	58	40	40	42	74		58
15 Minutes							
Range	42–84	50–56	48–52	54–68	70–112		52–77
Average	72	53	51	59	92		68

could be demonstrated following the injection of desoxycorticosterone acetate, progesterone, diethylstilbestrol, or α-estradiol benzoate. In each instance, the minimum values fell within the range of the control group. In comparing the average values, it may be noted that only the values for estradiol were above the average for the control group at 5, 10, and 15 minutes, while the average values for the other hormones were consistently below those for the control observations. The findings in similar experiments on three rabbits in the last week of gestation were almost identical with those in the control group.

COMMENT

The present experiments demonstrate that in the dog the increased rate of passage of chloride into artificial peritoneal fluid, previously observed following injection of

desoxycorticosterone acetate or progesterone, can also be produced by administration of diethylstilbestrol, α-estradiol benzoate and testosterone propionate. It has also been demonstrated that other steroid hormones exert a sodium, chloride and water-retaining effect in dogs and human beings (3, 4). As previously indicated, our observations support the hypothesis that the effect of these agents may be part of a more generalized action upon membranes rather than being due entirely to renal factors.

Alpha-estradiol benzoate proved to be the most potent agent in its effect on the passage of chloride into the peritoneal fluid in dogs. It is of interest that Thorn and Harrop (4) also found that this hormone exerted the most marked salt retaining effect in the human; they noted that the order of effectiveness of other hormones in this respect was as follows: progesterone, estrone, pregnandiol, testosterone, and testosterone propionate. Thorn and Emerson (5) have also presented evidence that a premenstrual increase in certain of these hormones is frequently accompanied by salt retention and edema. Although no direct attempt was made to determine the minimum quantity of hormone capable of increasing the rate of diffusion of chloride, no significant change was observed in two female dogs in whom the ovaries were stimulated 24 hours earlier with 400 I.U. of an equine gonadotropin.

The failure to demonstrate an increased rate of passage of chloride into the peritoneum of rabbits following the administration of desoxycorticosterone acetate, progesterone, or diethylstilbestrol indicates the presence of an important species difference in this regard. It is interesting to observe in this connection that we have been unable to produce hypertension, edema, or other toxic symptoms (except muscular weakness) in rabbits by the prolonged administration of large doses of desoxycorticosterone acetate.[2]

The essentially normal findings in three pregnant rabbits are also of interest since an increase in various steroid hormones has been demonstrated during pregnancy in different species; however, we have not been able to demonstrate an increased estrogen level in the blood of the pregnant rabbit.

SUMMARY

The administration of α-estradiol benzoate, diethylstilbestrol and testosterone propionate will cause an increased rate of diffusion of chloride into the peritoneum of dogs similar to that which we have previously demonstrated with desoxycorticosterone acetate and progesterone. This response could not be obtained in rabbits similarly treated or in rabbits tested during pregnancy.

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[2] Unpublished data.

NOTES AND COMMENTS

EFFECT OF VITAMIN B COMPLEX DEFICIENCY ON INACTIVATION OF OVARIAN ESTROGEN IN THE LIVER

SOME YEARS AGO, in the course of another investigation, one of us (M. C. S.) castrated a series of adult female rats and transplanted one ovary to the spleen. Though the transplants became well-vascularized and appeared normal in every respect, estrous cycles failed to appear in many of these animals. This was before the rôle of the liver in the inactivation of the estrogenic steroids was understood; the observation was not published and the phenomenon was not investigated further at that time.

It has since been conclusively demonstrated that natural and modified estrogens and androgens are inactivated in the liver.[1] In addition to other data on this general problem, the experiments of Golden and Sevringhaus[2] have a specific bearing on the observation already mentioned. They transplanted rats' ovaries to the mesentery and to the axillae; estrous cycles did not occur in the animals with transplants in the portal circulation but did occur in those with transplants in the axillae.

Employing a technic developed by G. R. Biskind and Mark[3] in which pellets of crystalline estrogens or androgens are implanted in the spleen, M. S. and G. R. Biskind[4] demonstrated that deficiency of the vitamin B complex in rats inhibits the estrogen-inactivating mechanism of the liver. Castrate female rats on a normal diet remained anestrous when a pellet of estrone was implanted in the spleen. When the animals were placed on a vitamin B complex-free diet protracted estrus occurred.

These observations appeared useful for a further study of the failure of the ovary transplanted to the portal circulation to produce estrus. However, previous observations by Evans and Bishop[5] and by others[6–8] indicated that in vitamin B complex deficiency rats become anestrous. We were therefore interested in determining whether the mechanism for inactivation of estrogen in the liver may be significantly impaired at a stage of vitamin B deficiency when the ovary is still functional.

Fourteen adult female rats of the Sherman strain, weighing from 121 to 206 gm., were castrated and one ovary was transplanted to the spleen. After a brief period to permit recovery from the operation, daily vaginal smears were made. Seven of the rats were found to have estrous cycles; these were killed and at necropsy in every case adhesions were found between the spleen or omentum and other organs that would have permitted blood from the portal circulation to enter systemic vessels. The other 7 animals remained completely anestrous and after from four to five weeks were placed on a vitamin B complex-free diet.† At this time the animals weighed from 195 to 250 gm.

After periods varying from 3 to 25 days on the B-free diet, each of these rats showed one

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† Vitamin B complex-free diet, SMACO (sucrose, 68 per cent; vitamin-free casein, 18 per cent; vegetable oil, 8 per cent; USP salt mixture No. 2, 4 per cent; USP cod liver oil, 2 per cent).

or more estrual reactions. One had a period of estrus lasting 11 days; another remained in estrus for 6 days and had two irregular cycles in addition. In two animals only brief and incomplete estrual reactions occurred. Two others had a single normal cycle each after 22 and 25 days respectively on the diet. Another had a series of four normal cycles beginning on the 11th day of dietary depletion.

The experiment was terminated after 28 days on the vitamin B complex-free diet at which time the animals had lost, on an average, one-third of their body weight. The rats were killed and the contents of the peritoneal cavity were carefully examined. In none of the animals were there any adhesions, either to the spleen or to other organs in the portal circulation that might have permitted blood from the spleen to enter the systemic circulation without first flowing through the liver. The ovarian implants were examined under a binocular dissecting microscope and in every case were seen to be well-vascularized and to contain ripe follicles. No gross lesions were detected in any of the livers.

It is thus possible in the rat to attain a stage of deficiency of the vitamin B complex in which the ability of the liver to inactivate ovarian estrogen is markedly impaired at a time when the ovary itself is still functional. The protracted periods of estrus observed in two of the animals indicate either that ovarian function had been disturbed or that, owing to diminished inactivation in the liver, the small amount of estrogen which might normally be secreted during diestrus escaped destruction and thus was able to produce an estrual response.

Partial deficiency of factors of the vitamin B complex may have an important bearing on clinical syndromes in which there is an excess of free estrogen. For instance, the occurrence of menorrhagia and metrorrhagia in patients with cirrhosis of the liver has long been known. Writing on this disease in Osler's *Modern Medicine* in 1908, Dr. A. O. J. Kelly pointed out, "Menorrhagia and metrorrhagia occur particularly in the early stages."

Using a basal diet similar to the one employed in the present investigation but supplemented with thiamin and riboflavin, or with thiamin, riboflavin and pyridoxine, Gyorgy and Goldblatt[9] have produced cirrhosis of the liver in rats. This could be prevented by brewers' yeast or by choline. Patek and Post[10] have shown that hepatic cirrhosis occurring in human beings may be ameliorated by nutritional therapy, especially by sources of the vitamin B complex. That the estrogen-inactivating mechanism of the liver is partly or entirely destroyed in patients with hepatic cirrhosis is indicated in a study by Glass and his associates.[11] They found that in male patients with this disease, the estrogen in the urine appeared in an active (unconjugated) form. Gynecomastia and testicular atrophy were associated with this excess of free estrogen.

The study reported here indicates that the estrogen-inactivating function of the liver may be disturbed even in the absence of visible organic lesions in this organ. Further data of our own as well as other observations in the literature which have a bearing on this problem are discussed in another paper.[12]

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ASSOCIATION NOTICE

PROCEEDINGS OF THE TWENTY-FIFTH ANNUAL MEETING

THE TWENTY-FIFTH ANNUAL MEETING of the Association for the Study of Internal Secretions was held in Atlantic City, New Jersey, at the Seaside Hotel on May 2nd and 3rd, 1941.

Four scientific sessions were held, and fifty-three papers were presented.

The subscription dinner was held in the Ballroom of the Seaside Hotel on Friday evening. President Elmer L. Sevringhaus delivered the address, presenting a report on the Second Pan-American Congress on Endocrinology held in Montevideo in March, 1941. This was illustrated with slides and motion pictures in color.

The second E. R. Squibb & Sons' Award was made to Professor Philip E. Smith for his work on the relation of the anterior pituitary gland to reproduction.

COUNCIL MEETINGS AND BUSINESS MEETING

The first meeting of the Council was at 7:30 A.M. on May 2nd. Various items of business were transacted and the following persons were elected to membership in the Association.

Ralph G. Bonime, M.D., Brooklyn, N. Y.
Charles L. Buxton, M.D., New York City
Eugene J. Cohen, M.D., New York City
John S. Evans, Ph.D., Kalamazoo, Mich.
Anne P. Forbes, M.D., Boston, Mass.
Harold Freedman, M.D., St. Louis, Mo.
Carl S. Gydesen, M.D., Colorado Springs, Colo.
Meyer M. Harris, M.D., New York City
J. Gerhardt Johnson, Rockford, Ill.
E. Henry Keutmann, M.D., Rochester, N. Y.
Otto S. Kretschmer, M.D., Denver, Colo.
Martin Kutscher, M.D., New York City
L. I. Pugsley, Ph.D., Ottawa, Canada
N. O. Rothermich, M.D., Columbus, Ohio
A. E. Sevringhaus, Ph.D., New York City
Reginald A. Shipley, M.D., Cleveland, Ohio
Patricia Smith, M.D., Boston, Mass.
Mayo Soley, M.D., San Francisco, Calif.
Carlos F. Speroni, M.D., Buenos Aires, Argentina
Herbert J. Spoor, Ph.D., Columbus, Ohio
Robert H. Williams, M.D., Boston, Mass.

A second meeting of the Council was held at 7:30 A.M. on May 3rd.

The general business meeting was held at 5:00 P.M. on May 3rd. The report of

the election tellers showed that the following officers were elected for the year 1941–1942.

President-Elect: E. Kost Shelton
Vice-President: E. M. K. Geiling
Secretary-Treasurer: Henry H. Turner
Members of the Council (three years): Elmer L. Sevringhaus
Roy G. Hoskins
Earl T. Engle
Members of the Publication Board (three years): C. N. H. Long
W. O. Thompson

A resolution of thanks was extended to Dr. and Mrs. Matthew Molitch for their excellent arrangements for the meeting.

A vote of thanks was extended to the management of the Seaside Hotel for their hospitality.

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